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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :	A1	(11) International Publication Number: WO 97/23614 (43) International Publication Date: 3 July 1997 (03.07.97)
C12N 15/12, C07K 14/715, C12N 5/10, A01K 67/027, C07K 19/00, C12N 15/62, C07K 16/28, 1/107, C12Q 1/68, G01N 33/50, 33/566, A61K 38/17, 48/00, C12N 1/21 // (C12N 1/21, C12R 1:19)		
(11) International Application Number: PCT/US96/20621		Crest Circle, Westlake Village, CA (US). CHANG, Ming-Shi [US/US]; 736 Calle Las Colinas, Newbury Park, CA (US).
(22) International Filing Date: 20 December 1996 (20.12.96)		
(30) Priority Data: 08/577,788 22 December 1995 (22.12.95) US 08/706,945 3 September 1996 (03.09.96) US		(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 De Havilland Drive, Thousand Oaks, CA 91320-1789 (US).
(60) Parent Application or Grant		
(63) Related by Continuation US 08/706,945 (CIP) Filed on 3 September 1996 (03.09.96)		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
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(54) Title: OSTEOPROTEGERIN

(57) Abstract

The present invention discloses a secreted polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily and is involved in the regulation of bone metabolism. Also disclosed are nucleic acids encoding osteoprotegerin, polypeptides, recombinant vectors and host cells for expression, antibodies which bind OPG, and pharmaceutical compositions. The polypeptides are used to treat bone diseases characterized by increased resorption such as osteoporosis.

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OSTEOPROTEGERIN

Field of the Invention

The invention relates generally to
5 polypeptides involved in the regulation of bone
metabolism. More particularly, the invention relates to
a novel polypeptide, termed osteoprotegerin, which is a
member of the tumor necrosis factor receptor
superfamily. The polypeptide is used to treat bone
10 diseases characterized by increased bone loss such as
osteoporosis.

Background of the Invention

Polypeptide growth factors and cytokines are
15 secreted factors which signal a wide variety of changes
in cell growth, differentiation, and metabolism, by
specifically binding to discrete, surface bound
receptors. As a class of proteins, receptors vary in
their structure and mode of signal transduction. They
20 are characterized by having an extracellular domain that
is involved in ligand binding, and cytoplasmic domain
which transmits an appropriate intracellular signal.
Receptor expression patterns ultimately determine which
cells will respond to a given ligand, while the
25 structure of a given receptor dictates the cellular
response induced by ligand binding. Receptors have been
shown to transmit intracellular signals via their
cytoplasmic domains by activating protein tyrosine, or
protein serine/threonine phosphorylation (e.g., platelet
30 derived growth factor receptor (PDGFR) or transforming
growth factor- β receptor-I (TGF β R-I), by stimulating
G-protein activation (e.g., β -adrenergic receptor), and
by modulating associations with cytoplasmic signal

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transducing proteins (e.g., TNFR-1 and Fas/APO) (Heldin, Cell 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, et al. Cell 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of these receptors (Chen et al., Chemistry 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNF α . (Goeddel et al. Cold Spring Harbor Symp. Quart. Biol. 51, 597-609 (1986); Nagata et al. Science 267, 1449-1456 (1995)). TNF α binds to distinct, but closely related receptors, TNFR-1 and TNFR-2. TNF α produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler et al. Ann. Rev. Biochem. 57, 505-518 (1988)).

TNF α is believed to mediate acute and chronic inflammatory responses (Beutler et al. Ann. Rev. Biochem. 57, 505-508 (1988)). Systemic delivery of TNF α induces toxic shock and widespread tissue necrosis. Because of this, TNF α may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda et al. Cell 75, 1169-1178 (1993)), is associated with autoimmunity (Fisher et al. Cell 81, 935-946 (1995)), while overproduction of FasL may be implicated in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects of many disease states, which suggests that agents that

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neutralize the activity of these ligands would have therapeutic value. Soluble TNFR-1 receptors, and antibodies that bind TNF α , have been tested for their ability to neutralize systemic TNF α (Loetscher et al. 5 Cancer Cells 3(6), 221-226 (1991)). A naturally occurring form of a secreted TNFR-1 mRNA was recently cloned, and its product tested for its ability to neutralize TNF α activity *in vitro* and *in vivo* (Kohno et al. PNAS USA 87, 8331-8335 (1990)). The ability of this 10 protein to neutralize TNF α suggests that soluble TNF receptors function to bind and clear TNF thereby blocking the cytotoxic effects on TNFR- bearing cells.

An object of the invention to identify new members of the TNFR super family. It is anticipated 15 that new family members may be transmembrane proteins or soluble forms thereof comprising extracellular domains and lacking transmembrane and cytoplasmic domains. We have identified a new member of the TNFR superfamily which encodes a secreted protein that is closely related 20 to TNFR-2. By analogy to soluble TNFR-1, the TNFR-2 related protein may negatively regulate the activity of its ligand, and thus may be useful in the treatment of certain human diseases.

25 Summary of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily has been identified from a fetal rat intestinal cDNA library. A full-length cDNA clone was obtained and sequenced. Expression of the rat 30 cDNA in a transgenic mouse revealed a marked increase in bones density, particularly in long bones, pelvic bone and vertebrae. The polypeptide encoded by the cDNA is termed Osteoprotegerin (OPG) and plays a role in promoting bone accumulation.

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The invention provides for nucleic acids encoding a polypeptide having at least one of the biological activities of OPG. Nucleic acids which hybridize to nucleic acids encoding mouse, rat or human OPG as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO: 122), and 9C-9D (SEQ ID NO: 124) are also provided. Preferably, OPG is mammalian OPG and more preferably is human OPG. Recombinant vectors and host cells expressing OPG are also encompassed as are methods of producing recombinant OPG. Antibodies or fragments thereof which specifically bind the polypeptide are also disclosed.

Methods of treating bone diseases are also provided by the invention. The polypeptides are useful for preventing bone resorption and may be used to treat any condition resulting in bone loss such as osteoporosis, hypercalcemia, Paget's disease of bone, and bone loss due to rheumatoid arthritis or osteomyelitis, and the like. Bone diseases may also be treated with anti-sense or gene therapy using nucleic acids of the invention. Pharmaceutical compositions comprising OPG nucleic acids and polypeptides are also encompassed.

25 Description of the Figures

Figure 1. A. FASTA analysis of novel EST LORF. Shown is the deduced FRI-1 amino acid sequence aligned to the human TNFR-2 sequence. B. Profile analysis of the novel EST LORF shown is the deduced FRI-1 amino acid sequence aligned to the TNFR-profile. C. Structural view of TNFR superfamily indicating region which is homologous to the novel FRI-1.

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Figure 2. Structure and sequence of full length rat OPG gene, a novel member of the TNFR superfamily. A. Map of pMOB-B1.1 insert. Box indicates position of LORF within the cDNA sequence (bold line). Black box 5 indicates signal peptide, and gray ellipses indicate position of cysteine-rich repeat sequences. B, C. Nucleic acid and protein sequence of the Rat OPG cDNA. The predicted signal peptide is underlined, and potential sites of N-linked glycosylation are indicated 10 in bold, underlined letters. D, E. Pileup sequence comparison (Wisconsin GCG Package, Version 8.1) of OPG with other members of the TNFR superfamily, fas (SEQ ID NO:128); tnfr1 (SEQ ID NO: 129); sfu-t2 (SEQ ID NO:130); tnfr2 (SEQ ID NO:131); cd40 (SEQ ID NO:132); osteo (SEQ 15 ID NO:133); ngfr (SEQ ID NO:134); ox40 (SEQ ID NO:135); 41bb (SEQ ID NO:136).

Figure 3. PepPlot analysis (Wisconsin GCG Package, Version 8.1) of the predicted rat OPG protein sequence.

20 A. Schematic representation of rat OPG showing hydrophobic (up) and hydrophilic (down) amino acids. Also shown are basic (up) and acidic (down) amino acids.

B. Display of amino acid residues that are beta-sheet forming (up) and beta-sheet breaking (down) as defined by 25 Chou and Fasman (Adv. Enz. 47, 45-147 (1948)). C. Display of propensity measures for alpha-helix and beta-sheet (Chou and Fasman, ibid). Curves above 1.00 show propensity for alpha-helix or beta-sheet structure. Structure may terminate in regions of protein where 30 curves drop below 1.00. D. Display of residues that are alpha-forming (up) or alpha-breaking (down). E. Display of portions of the protein sequence that resemble sequences typically found at the amino end of alpha and beta structures (Chou and Fasman, ibid). F. Display of

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portions of the protein sequence that resemble sequences typically found at the carboxyl end of alpha and beta structures (Chou and Fasman, *ibid*). G. Display of portions of the proteins sequence typically found in 5 turns (Chou and Fasman, *ibid*) H. Display of the helical hydrophobic moment (Eisenberg et al. Proc. Natl. Acad. Sci. USA 81, 140-144 (1984)) at each position in the sequence. I. Display of average hydrophathy based upon Kyte and Doolittle (J. Mol. Biol. 157, 105-132 (1982)) 10 and Goldman et al. (reviewed in Ann. Rev. Biophys. Biophys. Chem. 15, 321-353 (1986)).

Figure 4. mRNA expression patterns for the OPG cDNA in 15 human tissues. Northern blots were probed with a 32P-labeled rat cDNA insert (A, left two panels), or with the human cDNA insert (B, right panel).

Figure 5. Creation of transgenic mice expressing the 20 OPG cDNA in hepatocytes. Northern blot expression of HE-OPG transgene in mouse liver.

Figure 6. Increase in bone density in OPG transgenic mice. Panel A-F. Control Mice. G-J, OPG expressing 25 mice. At necropsy, all animals were radiographed and photographs prepared. In A-F, the radiographs of the control animals and the one transgenic non-expressor (#28) are shown. Note that the bones have a clearly defined cortex and a lucent central marrow cavity. In contrast, the OPG (G-J) animals have a poorly defined 30 cortex and increased density in the marrow zone.

Figure 7. Increase in trabecular bone in OPG transgenic mice. A-D. Representative photomicrographs of bones from control animals. In A and B, low (4X, 10X) power

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images of the femurs are shown (Masson Trichrome stain).
Stains for tartrate resistant acid phosphatase (TRAP)
demonstrate osteoclasts (see arrows) both resorbing
cartilage (C) and trabecular bone (D). Note the
5 flattened appearance of osteoclasts on trabecular bone.
E-H. Representative photomicrographs of bones from
OPG-expressing animals. In E and F, low (4X, 10X) power
images of the femurs are shown (Masson Trichrome stain).
The clear region is the growth plate cartilage, blue
10 stained area is bone, and the red area is marrow. Note
that in contrast to the controls, the trabecular bone
has not been resorbed resulting in the absence of the
usual marrow cavity. Also, the resulting trabeculae have
a variegated appearance with blue and clear areas. The
15 clear areas are remnants of growth plate cartilage that
have never been remodelled. Based on TRAP stains, these
animals do have osteoclasts (see arrows) at the growth
plate (G), which may be reduced in number. However, the
surfaces of the trabeculae away from the growth plate
20 are virtually devoid of osteoclasts (H), a finding that
stands in direct contrast with the control animals (see
D).

Figure 8. HE-OPG expressors do not have a defect in
25 monocyte-macrophage development. One cause for
osteopetrosis in mice is defective M-CSF production due
to a point mutation in the M-CSF gene. This results in
a marked deficit of circulating and tissue based
macrophages. The peripheral blood of OPG expressors
30 contained monocytes as assessed by H1E analysis. To
affirm the presence of tissue macrophages,
immunohistochemistry was performed using F480 antibodies,
which recognize a cell surface antigen on murine
macrophages. A and C show low power (4X)

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photomicrographs of the spleens from normal and CRI overexpressors. Note that both animals have numerous F480 positive cells. Monocyte-macrophages were also present in the marrow of normal (B) and HE-OPG 5 overexpressors (D) (40X).

Figure 9. Structure and sequence of mouse and human OPG cDNA clones. A, B. Mouse cDNA and protein sequence. C, D. Human cDNA and protein sequence. The predicted 10 signal peptides are underlined, and potential sites of N-linked glycosylation are indicated in bold. E, F. Sequence alignment and comparison of rat, mouse and human OPG amino acid sequences.

Figure 10. Comparison of conserved sequences in extracellular domain of TNFR-1 and human OPG. PrettyPlot (Wisconsin GCG Package, Version 8.1) of the TNFR1 and OPG alignment described in example 6. Top 15 line, human TNFR1 sequences encoding domains 1-4. Bottom line, human OPG sequences encoding domains 1-4. Conserved residues are highlighted by rectangular boxes.

Figure 11. Three-dimensional representation of human OPG. Side-view of the Molescript display of the 25 predicted 3-dimensional structure of human OPG residues 25 through 163, (wide line), co-crystallized with human TNF β (thin line). As a reference for orientation, the bold arrows along the OPG polypeptide backbone are pointing in the N-terminal to C-terminal direction. The 30 location of individual cysteine residue side chains are inserted along the polypeptide backbone to help demonstrate the separate cysteine-rich domains. The TNF β molecule is aligned as described by Banner et al. (1993).

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Figure 12. Structure of OPG cysteine-rich domains. Alignment of the human (top line SEQ ID NO:136) and mouse (bottom line) OPG amino acid sequences highlighting the predicted domain structure of OPG. The polypeptide is divided into two halves; the N-terminus (A), and C-terminus (B). The N-terminal half is predicted to contain four cysteine rich domains (labeled 1-4). The predicted intrachain disulfide bonds are indicated by bold lines, labeled "SS1", "SS2", or "SS3". Tyrosine 28 and histidine 75 (underlined) are predicted to form an ionic interaction. Those amino acids predicted to interact with an OPG ligand are indicated by bold dots above the appropriate residue. The cysteine residues located in the C-terminal half of OPG are indicated by rectangular boxes.

Figure 13. Expression and secretion of full length and truncated mouse OPG-Fc fusion proteins. A. Map indicating points of fusion to the human IgG1 Fc domain are indicated by arrowheads. B. Silver stain of a SDS-polyacrylamide gel of conditioned media obtained from cells expressing either F1.Fc (Full length OPG fused to Fc at Leucine 401) or CT.Fc (Carboxy-terminal truncated OPG fused to Fc at threonine 180) fusion protein expression vectors. Lane 1, parent pCEP4 expression vector cell line; Lane 2, F1.Fc vector cell line; Lane 3, CT.Fc vector cell line. C. Western blot of conditioned media obtained from F1.Fc and CT.Fc fusion protein expression vectors probed with anti-human IgG1 Fc domain (Pierce). Lane 1, parent pCEP4 expression vector cell line; Lane 2, F1.Fc vector cell line; Lane 3, CT.Fc vector cell line.

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Figure 14. Expression of human OPG in E. coli. A. Construction of a bacterial expression vector. The LORF of the human OPG gene was amplified by PCR, then joined to a oligonucleotide linker fragment (top strand is SEQ 5 ID NO:137; bottom strand is SEQ ID NO:127), and ligated into pAMG21 vector DNA. The resulting vector is capable of expressing OPG residues 32-401 linked to a N-terminal methionine residue. B SDS-PAGE analysis of uninduced and induced bacterial harboring the pAMG21-human OPG - 10 32-401 plasmid. Lane 1, MW standards; lane 2, uninduced bacteria; lane 3, 30°C induction; lane 4, 37°C induction; lane 5, whole cell lysate from 37°C induction; lane 6, soluble fraction of whole cell lysate; lane 7, insoluble fraction of whole cell lysate; 15 lane 8, purified inclusion bodies obtained from whole cell lysate.

Figure 15. Analysis of recombinant murine OPG produced in CHO cells by SDS-PAGE and western blotting. An equal amount of CHO conditioned media was applied to each lane shown, and was prepared by treatment with either 20 reducing sample buffer (left lane), or non-reducing sample buffer (right lane). After electrophoresis, the resolved proteins were transferred to a nylon membrane, 25 then probed with anti-OPG antibodies. The relative positions of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 16. Pulse-chase analysis of recombinant murine 30 OPG produced in CHO cells. CHO cells were pulse-labeled with ^{35}S -methionine/cysteine, then chased for the indicated time. Metabolically labeled cultures were separated into both conditioned media and cells, and detergent extracts were prepared from each, clarified,

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then immunoprecipitated with anti-OPG antibodies. The immunoprecipitates were resolved by SDS-PAGE, and exposed to film. Top left and right panels; samples analyzed under non-reducing conditions. Lower left and 5 right panels; samples analyzed under reducing conditions. Top and bottom left panels; Cell extracts. Top and bottom right panels; Conditioned media extracts. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

10

Figure 17. Expression of OPG in the CTLL-2 cell line. Serum-free conditioned media from CTLL-2 cells and CHO-mu OPG [1-401] transfected cells was prepared, concentrated, then analyzed by non-reducing SDS-PAGE and 15 western blotting. Left lane; CTLL-2 conditioned media. Right lane; CHO-muOPG conditioned media. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

20

Figure 18. Detection of OPG expression in serum samples and liver extracts obtained from control and OPG transgenic mice. Transgenic mice were constructed as described in Example 4. OPG expression was visualized after SDS-PAGE followed by Western blotting using 25 anti-OPG antibodies.

30

Figure 19. Effects of huOPG [22-401]-Fc fusion protein on osteoclast formation *in vitro*. The osteoclast forming assay was performed as described in Example 11A in the absence (control) or presence of the indicated amounts of huOPG [22-401]-Fc fusion. Osteoclast formation was visualized by histochemical staining for tartrate acid phosphatase (TRAP). A. OPG added to 100 ng/ml. D. OPG added to 0.1 ng/ml. E. OPG added to

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0.01 ng/ml. F. OPG added to 0.001 ng/ml. G. Control. No OPG added.

Figure 20. Decrease in osteoclast culture TRAP activity
5 with increasing amounts of OPG. Indicated concentrations of huOPG [22-401]-Fc fusion protein were added to osteoclast forming assay and TRAP activity quantitated as described in Example 11A.

10 Figure 21. Effect of OPG on a terminal stage of osteoclast differentiation. huOPG [22-401]-Fc fusion was added to the osteoclast forming assay during the intermediate stage of osteoclast maturation (days 5-6; OPG-CTL) or during the terminal stage of osteoclast 15 maturation (days 7-15; CTL-OPG). TRAP activity was quantitated and compared with the activity observed in the absence of OPG (CTL-CTL) in the presence of OPG throughout (OPG-OPG).

20 Figure 22. Effects of IL-1 β , IL-1 α and OPG on blood ionized calcium in mice. Levels of blood ionized calcium were monitored after injection of IL-1 β alone, IL-1 α alone, IL-1 β plus muOPG [22-401]-Fc, IL-1 α plus MuOPG [22-401]-Fc, and muOPG [22-401]-Fc alone. Control 25 mice received injections of phosphate buffered saline (PBS) only. IL-1 β experiment shown in A; IL-1 α experiment shown in B.

30 Figure 23. Effects of OPG on calvarial osteoclasts in control and IL1-treated mice. Histological methods for analyzing mice calvarial bone samples are described in Example 11B. Arrows indicate osteoclasts present in day 2-treated mice. Calvarial samples of mice receiving four PBS injections daily (A), one injection of IL-1 and

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three injections of PBS daily (B), one injection of PBS and three injections of OPG daily (C), one injection of IL-1 and three injections of OPG daily.

5 Figure 24. Radiographic analysis of bone accumulation in marrow cavity of normal mice. Mice were injected subcutaneously with saline (A) or muOPG [22-401]-Fc fusion (5mg/kg/d) for 14 days (B) and bone density determined as described in Example 11C.

10

Figure 25. Histomorphometric analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C.

15

Figure 26. Histology analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C.

20 A. Saline injection B. Injection of muOPG [22-401]-Fc fusion.

25 Figure 27. Activity of OPG administered to ovariectomized rats. In this two week experiment the trend to reduced bone density appears to be blocked by OPG or other anti-resorptive therapies. DEXA measurements were taken at time of ovariectomy and at week 1 and week 2 of treatment. The results are expressed as % change from the initial bone density (Mean +/- SEM).

30

Figure 28. Bone density in the femoral metaphysis, measured by histomorphometric methods, tends to be lower in ovariectomized rats (OVX) than sham operated animals (SHAM) 17 days following ovariectomy. This effect was

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blocked by OPG-Fc, with OPG-Fc treated ovariectomized rats (OVX+OPG) having significantly higher bone density than vehicle treated ovariectomized rats (OVX). (Mean +/- SEM).

5

Detailed Description of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily was identified as an expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library. The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in Examples 1 and 6. The rat, mouse and human genes are shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), respectively. All three sequences showed strong similarity to the extracellular domains of TNFR family members. None of the full-length cDNA clones isolated encoded transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human gene spanning nucleotides 1200-1353 shown in Figure 9D was deposited in the Genbank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat, mRNA expression was detected in kidney, liver, placenta and heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the same tissues along with lymph node, thymus, spleen and appendix.

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The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long bones (femurs), vertebrae and flat bones (pelvis).
5 Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and resorption which has led to a marked accumulation of bone and cartilage.
10 A decrease in the number of trabecular osteoclasts in the bones of OPG expressor animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent bone resorption, a process mediated by osteoclasts. In view of the activity in
15 transgenic expressors, the TNFR-related proteins described herein are termed OPGs.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse OPG in 293 cells and human OPG in E. coli is
20 described in Examples 7 and 8. Mouse OPG was produced as an Fc fusion which was purified by Protein A affinity chromatography. Also described in Example 7 is the expression of full-length and truncated human and mouse OPG polypeptides in CHO and 293 cells either as fusion
25 polypeptides to the Fc region of human IgG1 or as unfused polypeptides. The expression of full-length and truncated human and mouse OPGs in E. coli either as Fc fusion polypeptides or as unfused polypeptides is described in Example 8. Purification of recombinantly
30 produced mammalian and bacterial OPG is described in Example 10.

The biological activity of OPG was determined using an in vitro osteoclast maturation assay, an in vivo model of interleukin-1 (IL-1) induced

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hypercalcemia, and injection studies of bone density in normal mice (see Example 11). The following OPG recombinant proteins produced in CHO or 293 cells demonstrated activity in the in *E. coli* osteoclast 5 maturation assay: muOPG [22-185]-Fc, muOPG [22-194]-Fc, muOPG [22-401]Fc, muOPG [22-401], huOPG [22-201]-Fc, huOPG [22-401]-Fc. muOPG [22-180]-Fc produced in CHO cells and huOPG met[32-401] produced in *E. coli* did not demonstrate activity in the in vitro assay.

10 OPG from several sources was produced as a dimer and to some extent as a higher multimer. Rat OPG [22-401] produced in transgenic mice, muOPG [22-401] and huOPG [22-401] produced as a recombinant polypeptide in CHO cells, and OPG expressed as a naturally occurring 15 product from a cytotoxic T cell line were predominantly dimers and trimers when analyzed on nonreducing SDS gels (see Example 9). Truncated OPG polypeptides having deletions in the region of amino acids 186-401 (e.g., OPG [1-185] and OPG [1-194]) were predominantly 20 monomeric suggesting that the region 186-401 may be involved in self-association of OPG polypeptides. However, huOPG met[32-401] produced in *E. coli* was largely monomeric.

25 OPG may be important in regulating bone resorption. The protein appears to act as a soluble receptor of the TNF family and may prevent a receptor-ligand interaction involved in the osteolytic pathway. One aspect of the regulation appears to be a reduction in the number of osteoclasts.

30

Nucleic Acids

The invention provides for an isolated nucleic acid encoding a polypeptide having at least one of the biological activities of OPG. As described herein, the

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biological activities of OPG include, but are not limited to, any activity involving bone metabolism and in particular, include increasing bone density. The nucleic acids of the invention are selected from the
5 following:

- a) the nucleic acid sequences as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) or complementary strands thereof;
- b) the nucleic acids which hybridize under
10 stringent conditions with the polypeptide-encoding region in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124); and
- c) nucleic acids which hybridize under stringent conditions with nucleotides 148 through 337 inclusive as
15 shown in Figure 1A.
- d) the nucleic acid sequences which are degenerate to the sequences in (a) and (b).

The invention provides for nucleic acids which encode rat, mouse and human OPG as well as nucleic acid
20 sequences hybridizing thereto which encode a polypeptide having at least one of the biological activities of OPG. Also provided for are nucleic acids which hybridize to a rat OPG EST encompassing nucleotides 148-337 as shown in Figure 1A. The conditions for hybridization are
25 generally of high stringency such as 5xSSC, 50% formamide and 42°C described in Example 1 of the specification. Equivalent stringency to these conditions may be readily obtained by adjusting salt and organic solvent concentrations and temperature. The
30 nucleic acids in (b) encompass sequences encoding OPG-related polypeptides which do not undergo detectable hybridization with other known members of the TNF receptor superfamily. In a preferred embodiment, the nucleic acids are as shown in Figures 2B-2C (SEQ ID

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NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124).

The length of hybridizing nucleic acids of the invention may be variable since hybridization may occur 5 in part or all of the polypeptide-encoding regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), and may also occur in adjacent noncoding regions. Therefore, hybridizing nucleic acids may be truncations or extensions of the 10 sequences shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124). Truncated or extended nucleic acids are encompassed by the invention provided they retain one or more of the biological properties of OPG. The hybridizing nucleic 15 acids may also include adjacent noncoding regions which are 5' and/or 3' to the OPG coding region. The noncoding regions include regulatory regions involved in OPG expression, such as promoters, enhance, translational initiation sites, transcription 20 termination sites and the like.

Hybridization conditions for nucleic acids are described in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)

25 DNA encoding rat OPG was provided in plasmid pMO-B1.1 deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under ATCC accession no. 69970. DNA encoding mouse OPG was provided in plasmid pRcCMV-murine OPG deposited with the 30 American Type Culture Collection, Rockville, MD on December 27, 1995 under accession no. 69971. DNA encoding human OPG was provided in plasmid pRcCMV-human OPG deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under

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accession no. 69969. The nucleic acids of the invention will hybridize under stringent conditions to the DNA inserts of ATCC accession nos. 69969, 69970, and 69971 and have at least one of the biological activities of

5 OPG.

Also provided by the invention are derivatives of the nucleic acid sequences as shown in Figures 2B, 9A and 9B. As used herein, derivatives include nucleic acid sequences having addition, substitution, insertion 10 or deletion of one or more residues such that the resulting sequences encode polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting polypeptide has the activity of OPG. The nucleic acid derivatives 15 may be naturally occurring, such as by splice variation or polymorphism, or may be constructed using site-directed mutagenesis techniques available to the skilled worker. One example of a naturally occurring variant of OPG is a nucleic acid encoding a lys to asn change at 20 residue 3 within the leader sequence (see Example 5). It is anticipated that nucleic acid derivatives will encode amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other derivatives include a nucleic acid encoding a 25 membrane-bound form of OPG having an extracellular domain as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) along with transmembrane and cytoplasmic domains.

In one embodiment, derivatives of OPG include 30 nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the carboxy terminus. Nucleic acids encoding OPG may have from 1 to 216 amino acids deleted from the carboxy terminus. Optionally, an antibody Fc region may extend from the new carboxy

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terminus to yield a biologically active OPG-Fc fusion polypeptide. (see Example 11). In preferred embodiments, nucleic acids encode OPG having the amino acid sequence from residues 22-185, 22-189, 22-194 or 5 22-201 (using numbering in Figure 9E-F) and optionally, encoding an Fc region of human IgG.

Also included are nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the amino terminus. Truncated forms 10 include those lacking part or all the 21 amino acids comprising the leader sequence. Additionally, the invention provides for nucleic acids encoding OPG having from 1 to 10 amino acids deleted from the mature amino terminus (at residue 22) and, optionally, having from 1 15 to 216 amino acids deleted from the carboxy terminus (at residue 401). Optionally, the nucleic acids may encode a methionine residue at the amino terminus. Examples of such OPG truncated polypeptides are described in Example 8.

20 Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing OPG. In humans, tissue sources for OPG include kidney, liver, placenta and 25 heart. Genomic DNA encoding OPG is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to 30 reconstitute part or all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of interferon genes). RNA is obtained most easily by prokaryotic expression

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secreted polypeptides which may be recovered from the growth medium.

Vectors for the expression of OPG contain at a minimum sequences required for vector propagation and for expression of the cloned insert. These sequences include a replication origin, selection marker, promoter, ribosome binding site, enhancer sequences, RNA splice sites and transcription termination site. Vectors suitable for expression in the aforementioned host cells are readily available and the nucleic acids of the invention are inserted into the vectors using standard recombinant DNA techniques. Vectors for tissue-specific expression of OPG are also included. Such vectors include promoters which function specifically in liver, kidney or other organs for production in mice, and viral vectors for the expression of OPG in targeted human cells.

Using an appropriate host-vector system, OPG is produced recombinantly by culturing a host cell transformed with an expression vector containing nucleic acid sequences encoding OPG under conditions such that OPG is produced, and isolating the product of expression. OPG is produced in the supernatant of transfected mammalian cells or in inclusion bodies of transformed bacterial host cells. OPG so produced may be purified by procedures known to one skilled in the art as described below. The expression of OPG in mammalian and bacterial host systems is described in Examples 7 and 8. Expression vectors for mammalian hosts are exemplified by plasmids such as pDSR α described in PCT Application No. 90/14363. Expression vectors for bacterial host cells are exemplified by plasmids pAMG21 and pAMG22-His described in Example 8. Plasmid pAMG21 was deposited with the American Type

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vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Nucleic acid sequences of the invention are used for the detection of OPG sequences in biological samples in order to determine which cells and tissues are expressing OPG mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to OPG. Such screening is well within the capabilities of one skilled in the art using appropriate hybridization conditions to detect homologous sequences. The nucleic acids are also useful for modulating the expression of OPG levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity (see Example 3).

Vectors and Host Cells

Expression vectors containing nucleic acid sequences encoding OPG, host cells transformed with said vectors and methods for the production of OPG are also provided by the invention. An overview of expression of recombinant proteins is found in Methods of Enzymology v. 185, Goeddel, D.V. ed. Academic Press (1990).

Host cells for the production of OPG include prokaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression. Preferred mammalian host cells include COS, CHO^{d-}, 293, CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for OPG activity. Mammalian expression allows for the production of

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Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98113. Plasmid pAMG22-His was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98112. It is

5 anticipated that the specific plasmids and host cells described are for illustrative purposes and that other available plasmids and host cells could also be used to express the polypeptides.

10 The invention also provides for expression of OPG from endogenous nucleic acids by in vivo or ex vivo recombination events to allow modulation of OPG from the host chromosome. Expression of OPG by the introduction of exogenous regulatory sequences (e.g. promoters or enhancers) capable of directing the production of OPG
15 from endogenous OPG coding regions is also encompassed. Stimulation of endogenous regulatory sequences capable of directing OPG production (e.g. by exposure to transcriptional enhancing factors) is also provided by the invention.

20

Polypeptides

The invention provides for OPG, a novel member of the TNF receptor superfamily, having an activity associated with bone metabolism and in particular having
25 the activity of inhibiting bone resorption thereby increasing bone density. OPG refers to a polypeptide having an amino acid sequence of mouse, rat or human OPG or a derivative thereof having at least one of the biological activities of OPG. The amino acid sequences
30 of rat, mouse and human OPG are shown in Figures 2B-2C (SEQ ID NO:121), 9A-9B (SEQ ID NO:123), and 9C-9D (SEQ ID NO:125) respectively. A derivative of OPG refers to a polypeptide having an addition, deletion, insertion or substitution of one or more amino acids such that the

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resulting polypeptide has at least one of the biological activities of OPG. The biological activities of OPG include, but are not limited to, activities involving bone metabolism. Preferably, the polypeptides will have 5 the amino terminal leader sequence of 21 amino acids removed.

OPG polypeptides encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]-Fc fusion, rat [1-180]-Fc fusion, mouse [1-10 401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180], human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO:121 (rat), SEQ ID NO:123 (mouse) and SEQ ID NO:125 (human). Also 15 encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of OPG; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of OPG, in particular deletion of 20 the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain. In one embodiment, OPG has from 1 to about 216 amino acids deleted from the carboxy 25 terminus. In another embodiment, OPG has from 1 to about 10 amino acids deleted from the mature amino terminus (wherein the mature amino terminus is at residue 22) and, optionally, has from 1 to about 216 amino acids deleted from the carboxy terminus.

30 Additional OPG polypeptides encompassed by the invention include the following: human [22-180]-Fc fusion, human [22-201]-Fc fusion, human [22-401]-Fc fusion, mouse [22-185]-Fc fusion, mouse [22-194]-Fc fusion. These polypeptides are produced in mammalian

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host cells, such as CHO or 293 cells. Additional OPG polypeptides encompassed by the invention which are expressed in prokaryotic host cells include the following: human met[22-401], Fc-human met[22-401] fusion (Fc region is fused at the amino terminus of the full-length OPG coding sequence as described in Example 8), human met[22-401]-Fc fusion (Fc region fused to the full-length OPG sequence), Fc-mouse met[22-401] fusion, mouse met[22-401]-Fc fusion, human met[27-401], human met[22-185], human met[22-189], human met[22-194], human met[22-194] (P25A), human met[22-194] (P26A), human met[27-185], human met[27-189], human met[27-194], human met-arg-gly-ser-(his)₆[22-401], human met-lys[22-401], human met-(lys)₃[22-401], human met[22-401]-Fc (P25A),
10 human met[22-401] (P25A), human met[22-401] (P26A), human met[22-401] (P26D), mouse met[22-401], mouse met[27-401], mouse met[32-401], mouse met[27-180], mouse met[22-189], mouse met[22-194], mouse met[27-189], mouse met[27-194], mouse met-lys[22-401], mouse HEK[22-401] (A45T), mouse met-lys-(his)₇[22-401], mouse met-lys[22-401]-(his)₇ and mouse met[27-401] (P33E, G36S, A45P). It is understood that the above OPG polypeptides produced in prokaryotic host cells have an amino-terminal methionine residue, if such a residue is not indicated. In specific examples, OPG-Fc fusion were produced using a 227 amino acid region of human IgG1- γ 1 was used having the sequence as shown in Ellison et al. (Nuc. Acids Res. 10, 4071-4079 (1982)). However, variants of the Fc region of human IgG may also be used.

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20
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Analysis of the biological activity of carboxy-terminal OPG truncations fused to the human IgG1 Fc region indicates a portion of OPG of about 164 amino acids which is required for activity. This region encompasses amino acids 22-185, preferably those in

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Figure 9C-9D (SEQ ID NO:125), and comprises four cysteine-rich domains characteristic of the cysteine-rich domains of TNFR extracellular domains.

Using the homology between OPG and the 5 extracellular ligand binding domains of TNF receptor family members, a three-dimensional model of OPG was generated based upon the known crystal structure of the extracellular domain of TNFR-I (see Example 6). This model was used to identify those residues within OPG 10 which may be important for biological activity. Cysteine residues that are involved in maintaining the structure of the four cysteine-rich domains were identified. The following disulfide bonds were identified in the model: Domain 1: cys41 to cys54, cys44 15 to cys62, tyr23 and his 66 may act to stabilize the structure of this domain; Domain 2: cys65 to cys80, cys83 to cys98, cys87 to cys105; Domain 3: cys107 to cys118, cys124 to cys142; Domain 4: cys145 to cys160, cys166 to cys185. Residues were also identified which 20 were in close proximity to TNF β as shown in Figures 11 and 12A-12B. In this model, it is assumed that OPG binds to a corresponding ligand; TNF β was used as a model ligand to simulate the interaction of OPG with its ligand. Based upon this modeling, the following 25 residues in OPG may be important for ligand binding: glu34, lys43, pro66 to gln91 (in particular, pro66, his68, tyr69, tyr70, thr71, asp72, ser73, his76, ser77, asp78, glu79, leu81, tyr82, pro85, val86, lys88, glu90 and gln91), glu153 and ser155.

30 Alterations in these amino acid residues, either singly or in combination, may alter the biological activity of OPG. For example, changes in specific cysteine residues may alter the structure of individual cysteine-rich domains, whereas changes in

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residues important for ligand binding may affect physical interactions of OPG with ligand. Structural models can aid in identifying analogs which have more desirable properties, such as enhanced biological

5 activity, greater stability, or greater ease of formulation.

The invention also provides for an OPG multimer comprising OPG monomers. OPG appears to be active as a multimer (e.g., dimer, trimer or a higher 10 number of monomers). Preferably, OPG multimers are dimers or trimers. OPG multimers may comprise monomers having the amino acid sequence of OPG sufficient to promote multimer formation or may comprise monomers having heterologous sequences such as an antibody Fc 15 region. Analysis of carboxy-terminal deletions of OPG suggest that at least a portion of the region 186-401 is involved in association of OPG polypeptides. Substitution of part or all of the region of OPG amino acids 186-401 with an amino acid sequence capable of 20 self-association is also encompassed by the invention. Alternatively, OPG polypeptides or derivatives thereof may be modified to form dimers or multimers by site directed mutagenesis to create unpaired cysteine residues for interchain disulfide bond formation, by 25 photochemical crosslinking, such as exposure to ultraviolet light, or by chemical crosslinking with bifunctional linker molecules such as bifunctional polyethylene glycol and the like.

Modifications of OPG polypeptides are 30 encompassed by the invention and include post-translational modifications (e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked

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or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a result of prokaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, 5 fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Further modifications of OPG include chimeric proteins wherein OPG is fused to a heterologous amino acid sequence. The heterologous sequence may be any 10 sequence which allows the resulting fusion protein to retain the activity of OPG. The heterologous sequences include for example, immunoglobulin fusions, such as Fc fusions, which may aid in purification of the protein. A heterologous sequence which promotes association of 15 OPG monomers to form dimers, trimers and other higher multimeric forms is preferred.

The polypeptides of the invention are isolated and purified from other polypeptides present in tissues, cell lines and transformed host cells expressing OPG, or 20 purified from components in cell cultures containing the secreted protein. In one embodiment, the polypeptide is free from association with other human proteins, such as the expression product of a bacterial host cell.

Also provided by the invention are chemically 25 modified derivatives of OPG which may provide additional advantages such as increasing stability and circulating time of the polypeptide, or decreasing immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble 30 polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined

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positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 5 1kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other 10 sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a 15 therapeutic protein or analog).

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of 20 attachment methods available to those skilled in the art, e.g. EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20: 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, 25 polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino 30 group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulphydrl groups may also be used as a reactive group

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for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

5 One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion
10 of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation
15 (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemically modification may be accomplished by reductive
20 alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of
25 the protein at the N-terminus with a carbonyl group containing polymer is achieved.

Synthetic OPG dimers may be prepared by various chemical crosslinking procedures. OPG monomers may be chemically linked in any fashion that retains or
30 enhances the biological activity of OPG. A variety of chemical crosslinkers may be used depending upon which properties of the protein dimer are desired. For example, crosslinkers may be short and relatively rigid or longer and more flexible, may be biologically

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reversible, and may provide reduced immunogenicity or longer pharmacokinetic half-life.

In one example, OPG molecules are linked through the amino terminus by a two step synthesis (see Example 12). In the first step, OPG is chemically modified at the amino terminus to introduce a protected thiol, which after purification is deprotected and used as a point of attachment for site-specific conjugation through a variety of crosslinkers with a second OPG molecule. Amino-terminal crosslinks include, but are not limited to, a disulfide bond, thioether linkages using short-chain, bis-functional aliphatic crosslinkers, and thioether linkages to variable length, bifunctional polyethylene glycol crosslinkers (PEG "dumbbells"). Also encompassed by PEG dumbbell synthesis of OPG dimers is a byproduct of such synthesis, termed a "monobell". An OPG monobell consists of a monomer coupled to a linear bifunctional PEG with a free polymer terminus. Alternatively, OPG may be crosslinked directly through a variety of amine specific homobifunctional crosslinking techniques which include reagents such as: diethylenetriaminepentaacetic dianhydride (DTPA), p-benzoquinone (pBQ) or bis(sulfosuccinimidyl) suberate (BS³) as well as others known in the art. It is also possible to thiolate OPG directly with reagents such as iminothiolane in the presence of a variety of bifunctional, thiol specific crosslinkers, such as PEG bismaleimide, and achieve dimerization and/or dumbbells in a one step process.

A method for the purification of OPG from natural sources and from transfected host cells is also included. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The

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chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG antibody or biotin-streptavidin affinity complex and the like.

5

Antibodies

Also encompassed by the invention are antibodies specifically binding to OPG. Antigens for 10 the generation of antibodies may be full-length polypeptides or peptides spanning a portion of the OPG sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with OPG are known to one skilled in the art (see, for example, 15 Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies 20 also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary 25 determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the art. Also encompassed are human antibodies made in mice.

30

Anti-OPG antibodies of the invention may be used as an affinity reagent to purify OPG from biological samples (see Example 10). In one method, the antibody is immobilized on CNBr-activated Sepharose and a column of antibody-Sepharose conjugate is used to

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remove OPG from liquid samples. Antibodies are also used as diagnostic reagents to detect and quantitate OPG in biological samples by methods described below.

5 Pharmaceutical compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the polypeptide of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The term "therapeutically effective amount" means an amount which provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal or benzyl alcohol, and antioxidants such as ascorbic acid or sodium metabisulfite. Also encompassed are compositions comprising OPG modified with water soluble polymers to increase solubility or stability. Compositions may also comprise incorporation of OPG into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time. Specifically, OPG compositions may comprise incorporation into polymer matrices such as hydrogels, silicones, polyethylenes, ethylene-vinyl acetate copolymers, or biodegradable polymers. Examples of hydrogels include polyhydroxyalkylmethacrylates (p-HEMA), polyacrylamide, polymethacrylamide, polyvinylpyrrolidone, polyvinyl alcohol and various polyelectrolyte complexes. Examples of biodegradable polymers include polylactic acid (PLA), polyglycolic

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acid (PGA), copolymers of PLA and PGA, polyamides and copolymers of polyamides and polyesters. Other controlled release formulations include microcapsules, microspheres, macromolecular complexes and polymeric
5 beads which may be administered by injection.

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive
10 survey of component suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1980).

15 Compositions of the invention may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled
20 in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the OPG coding region to cells and tissues as part of an anti-sense or gene therapy regimen.
25

30 Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, osteocytes,

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osteoblasts and osteoclasts, are involved in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of bone tissue whereas osteoclasts are associated with resorption.

5 Resorption, or the dissolution of bone matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal tissue and in
10 resorption induced by hormones. For instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of
15 calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone formation and bone resorption. Peak bone mass occurs
20 after skeletal maturity prior to the fourth decade. Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts
25 earlier in females than males and is distinctly accelerated after menopause in some females (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels.
30 Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of

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osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in bone resorption with a normal rate of bone formation. About 25 to 30 percent of all white females in the 5 United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50 10 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been identified which may contribute to the condition. They include alteration in hormone levels accompanying aging 15 and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard the process. To date, however, an effective treatment 20 for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of OPG. The bone disorder may be any disorder characterized by a net bone loss (osteopenia or 25 osteolysis). In general, treatment with OPG is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption rate is above normal or to reduce bone resorption to below 30 normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with OPG include the following:

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Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of 5 osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.

Paget's disease of bone (osteitis deformans) in adults and juveniles

10 Osteomyelitis, or an infectious lesion in bone, leading to bone loss.

Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignancies (multiple myeloma, lymphoma and leukemia), idiopathic 15 hypercalcemia, and hypercalcemia associated with hyperthyroidism and renal function disorders.

20 Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic and renal diseases.

Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus and other conditions.

25 Bone loss due to rheumatoid arthritis.

Periodontal bone loss.

Osteolytic metastasis

It is understood that OPG may be used alone or 30 in conjunction with other factors for the treatment of bone disorders. In one embodiment, osteoprotegerin is used in conjunction with a therapeutically effective amount of a factor which stimulates bone formation. Such factors include but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12,

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- transforming growth factor- β (TGF- β) and TGF- β family members, interleukin-1 inhibitors, TNF α inhibitors, parathyroid hormone and analogs thereof, parathyroid related protein and analogs thereof, E series 5 prostaglandins, bisphosphonates (such as alendronate and others), and bone-enhancing minerals such as fluoride and calcium.

10 The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

15

EXAMPLE 1
Identification and isolation of the
rat OPG cDNA

20 Materials and methods for cDNA cloning and analysis are described in Maniatis et al, ibid. Polymerase chain reactions (PCR) were performed using a Perkin-Elmer 9600 thermocycler using PCR reaction mixture (Boehringer-Mannheim) and primer concentrations specified by the manufacturer. In general, 25-50 μ l 25 reactions were denatured at 94°C, followed by 20-40 cycles of 94°C for 5 seconds, 50-60°C for 5 seconds, and 72°C for 3-5 minutes. Reactions were then analyzed by gel electrophoresis as described in Maniatis et al., ibid.

30 A cDNA library was constructed using mRNA isolated from embryonic d20 intestine for EST analysis (Adams et al. Science 252, 1651-1656 (1991)). Rat embryos were dissected, and the entire developing small and large intestine removed and washed in PBS. Total

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cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi Anal. Biochem. 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA 5 preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the manufacturer's recommended procedures. A random primed cDNA library was prepared using the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md). The random cDNA 10 primer containing an internal Not I restriction site was used to initiate first strand synthesis and had the following sequence:

5'-AAAGGAAGGAAAAAGCGGCCGCTACANNNNNNNNT-3' (SEQ ID NO:1)

Not I

15 For the first strand synthesis three separate reactions were assembled that contained 2.5 µg of poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random primer. After second strand synthesis, the reaction products were separately extracted with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1 ratio), and 20 then ethanol precipitated. The double strand (ds) cDNA products of the three reactions were combined and ligated to the following ds oligonucleotide adapter:

25 5'-TCGACCCACGCGTCCG-3' (SEQ ID NO:2)

3'-GGGTGCGCAGGCP-5' (SEQ ID NO:3)

After ligation the cDNA was digested to completion with Not I, extracted with 30 phenol:chloroform:isoamyl (25:24:1) alcohol and ethanol precipitated. The resuspended cDNA was then size fractionated by gel filtration using premade columns provided with the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) as recommended by the manufacturer.

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The two fractions containing the largest cDNA products were pooled, ethanol precipitated and then directionally ligated into Not I and Sal I digested pMOB vector DNA (Strathmann et al, 1991). The ligated cDNA was
5 introduced into competent ElectroMAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) by electroporation. For automated sequence analysis approximately 10,000 transformants were plated on 20cm x 20cm agar plates containing ampicillin supplemented LB nutrient media.
10 The colonies that arose were picked and arrayed onto 96 well microtiter plates containing 200 ml of L-broth, 7.5% glycerol, and 50 µg/ml ampicillin. The cultures were grown overnight at 37°C, a duplicate set of microtiter plates were made using a sterile 96 pin
15 replicating tool, then both sets were stored at -80°C for further analysis. For full-length cDNA cloning approximately one million transformants were plated on 96 bacterial ampicillin plates containing about 10,000 clones each. The plasmid DNA from each pool was
20 separately isolated using the Qiagen Plasmid Maxi Kit (Qiagen Corp., Germany) and arrayed into 96 microtiter plates for PCR analyses.

To sequence random fetal rat intestine cDNA clones, glycerol stocks were thawed, and small aliquots
25 diluted 1:25 in distilled. Approximately 3.0 ul of diluted bacterial cultures were added to PCR reaction mixture (Boehringer-Mannheim) containing the following oligonucleotides:

30 5'-TGTAAAACGACGGCCAGT-3' (SEQ ID NO:4)
5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO:5)

The reactions were incubated in a thermocycler (Perkin-Elmer 9600) with the following cycle conditions:

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94°C for 2 minutes; 30 cycles of 94°C for 5 seconds, 50°C for 5 seconds, and 72°C for 3 minutes.; 72°C for 4 minutes. After incubation in the thermocycler, the reactions were diluted with 2.0 mL of water. The 5 amplified DNA fragments were further purified using Centricon columns (Princeton Separations) using the manufacturer's recommended procedures. The PCR reaction products were sequenced on an Applied Biosystems 373A 10 automated DNA sequencer using T3 primer (oligonucleotide 353-23; 5'-CAATTAACCCTCACTAAAGG-3') (SEQ ID NO:6) Taq dye-terminator reactions (Applied Biosystems) following the manufacturer's recommended procedures.

The resulting 5' nucleotide sequence obtained from randomly picked cDNA clones translated and then 15 compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson et al. *Meth. Enzymol.* 183, (1990)). Translated sequences were also analysed for the presence of a specific cysteine-rich protein motif found in all known 20 members of the tumor necrosis factor receptor (TNFR) superfamily (Smith et al. *Cell* 76, 959-962 (1994)), using the sequence profile method of Gribskov et al. (Proc. Natl. Acad. Sci. USA 83, 4355-4359 (1987)), as modified by Luethy et al. (Protein Science 3, 139-146 25 (1994)).

Using the FASTA and Profile search data, an EST, FRI-1 (Fetal Rat Intestine-1), was identified as a possible new member of the TNFR superfamily. FRI-1 contained an approximately 600 bp insert with a LORF of 30 about 150 amino acids. The closest match in the database was the human type II TNFR (TNFR-2). The region compared showed an ~43% homology between TNFR-2 and FRI-1 over this 150 aa LORF. Profile analysis using the first and second cysteine-rich repeats of the TNFR

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superfamily yielded a Z score of ~8, indicating that the FRI-1 gene possibly encodes a new family member. To deduce the structure of the FRI-1 product, the fetal rat intestine cDNA library was screened for full length 5 clones. The following oligonucleotides were derived from the original FRI-1 sequence:

5'-GCATTATGACCCAGAAACCGGAC-3' (SEQ ID NO:7)
5'-AGGTAGCGCCCTTCCTCACATTC-3' (SEQ ID NO:8)

10

These primers were used in PCR reactions to screen 96 pools of plasmid DNA, each pool containing plasmid DNA from 10,000 independent cDNA clones. Approximately 1 ug of plasmid pool DNA was amplified in 15 a PCR reaction mixture (Boehringer-Mannheim) using a Perkin-Elmer 96 well thermal cycler with the following cycle conditions: 2 min at 94°C, 1 cycle; 15 sec at 94°C, then 45 sec at 65°C, 30 cycles; 7 min at 65°C, 1 cycle. PCR reaction products were analysed by gel 20 electrophoresis. 13 out of 96 plasmid DNA pools gave rise to amplified DNA products with the expected relative molecular mass.

DNA from one positive pool was used to transform competent ElectroMAX DH10B *E. coli* (Gibco BRL, 25 Gaithersburg, MD) as described above. Approximately 40,000 transformants were plated onto sterile nitrocellulose filters (BA-85, Schleicher and Schuell), and then screened by colony hybridization using a 32P-dCTP labelled version of the PCR product obtained above. 30 Filters were prehybridized in 5X SSC, 50% deionized formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C. Filters were then hybridized in 5X SSC, 50% deionized formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml

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denatured salmon sperm DNA, and ~5 ng/ml of labelled probe for ~18 hours at 42°C. The filters were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 55°C, and finally in 0.5X SSC for 10-15 min at 55°C.

- 5 Hybridizing clones were detected following autoradiography, and then replated onto nitrocellulose filters for secondary screening. Upon secondary screening, a plasmid clone (pB1.1) was isolated, then amplified in L-broth media containing 100 ug/ml
10 ampicillin and the plasmid DNA obtained. Both strands of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA search of the public database to detect any existing sequence matches and/or similarities. No matches to any
15 known genes or EST's were found, although there was an approximate 45% similarity to the human and mouse TNFR-2 genes. A methionine start codon is found at bp 124 of the nucleotide sequence, followed by a LORF encoding 401 aa residues that terminates at bp 1327. The 401 aa
20 residue product is predicted to have a hydrophobic signal peptide of approximately 31 residues at its N-terminus, and 4 potential sites of N-linked glycosylation. No hydrophobic transmembrane spanning sequence was identified using the PepPlot program
25 (Wisconsin GCG package, version 8.1). The deduced 401 aa sequence was then used to search the protein database. Again, there were no existing matches, although there appeared to be a strong similarity to many members of the TNFR superfamily, most notably the
30 human and mouse TNFR-2. A sequence alignment of this novel protein with known members of the TNFR-superfamily was prepared using the Pileup program, and then modified by PrettyPlot (Wisconsin GCG package, version 8.1). This alignment shows a clear homology between the full

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length FRI-1 gene product and all other TNFR family members. The homologous region maps to the extracellular domain of TNFR family members, and corresponds to the three or four cysteine-rich repeats found in the ligand binding domain of these proteins. This suggested that the FRI-1 gene encoded a novel TNFR family member. Since no transmembrane spanning region was detected we predicted that this may be a secreted receptor, similar to TNFR-1 derived soluble receptors (Kohno et al. Proc. Natl. Acad. Sci. USA 87, 8331-8335 (1990)). Due to the apparent biological activity of the FRI-1 gene (*vide infra*), the product was named Osteoprotegerin (OPG).

15

EXAMPLE 2

OPG mRNA Expression Patterns in Tissues

Multiple human tissue northern blots (Clonetech) were probed with a ³²P-dCTP labelled FRI-1 PCR product to detect the size of the human transcript and to determine patterns of expression. Northern blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2-4 hr at 42°C. The blots were then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C. The blots were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15 min.

Using a probe derived from the rat gene, a predominant mRNA species with a relative molecular mass of about 2.4 kb is detected in several tissues, including kidney, liver, placenta, and heart. Highest

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levels are detected in the kidney. A large mRNA species of Mr 4.5 and 7.5 kb was detected in skeletal muscle and pancreas. In human fetal tissue, kidney was found to express relatively high levels of the 2.4 kb mRNA.

- 5 Using a human probe (vide infra), only the 2.4 kb transcript is detected in these same tissues. In addition, relatively high levels of the 2.4 kb transcript was detected in the lymph node, thymus, spleen and appendix. The size of the transcript
10 detected by both the rat and human Osteoprotegerin gene is almost identical to the length of the rat pB1.1 FRI-1 insert, suggesting it was a full length cDNA clone.

EXAMPLE 3

15 Systemic delivery of OPG in transgenic mice

The rat OPG clone pB1.1 was used as template to PCR amplify the coding region for subcloning into an ApoE-liver specific expression vector (Simonet et al. J. Clin. Invest. 94, 1310-1319 (1994), and PCT Application No. US94/11675 and co-owned U.S. Serial No. 08/221,767. The following 5' and 3' oligonucleotide primers were used for PCR amplification, respectively:

25 5'-GACTAGTCCCACAATGAACAAAGTGGCTGTG-3' (SEQ ID NO:9)
5'-ATAAGAATGCAGCCGCTAAACTATGAAACAGCCCAGTGACCATTG-3'
(SEQ ID NO:10)

30 The PCR reaction mixture (Boehringer-Mannheim) was treated as follows: 94°C for 1 minute, 1 cycle; 94°C for 20 sec, 62°C for 30 sec, and 74 C for 1 minute, 25 cycles. Following amplification, the samples were purified over Qiagen PCR columns and digested overnight with SpeI and NotI restriction enzymes. The digested

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products were extracted and precipitated and subcloned into the ApoE promoter expression vector. Prior to microinjecting the resulting clone, HE-OPG, it was sequenced to ensure it was mutation-free.

5 The HE-OPG plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid DNA was digested with XbaI and Ase I, and the 3.6 kb transgene insert was purified by gel electrophoresis. The purified fragment was diluted to a stock injection solution of 1 µg/ml in 5 mM Tris, pH 7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x BDF1-bred mice were injected essentially as described (Brinster et al., Proc. Natl. Acad. Sci. USA 82, 4338 (1985)), except that injection needles were beveled and siliconized before use. Embryos were cultured overnight in a CO₂ incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

20 Following term pregnancy, 49 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the human Apo E intron which was included in the expression vector. The oligos used for PCR amplification were:

5'- GCC TCT AGA AAG AGC TGG GAC-3' (SEQ ID NO:11)

5'- CGC CGT GTT CCA TTT ATG AGC-3' (SEQ ID NO:12)

30 The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were identified as PCR positive transgenic founders.

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At 8-10 weeks of age, five transgenic founders (2, 11, 16, 17, and 28) and five controls (1, 12, 15, 18, and 30) were sacrificed for necropsy and pathological analysis. Liver was isolated from the 5 remaining 4 founders by partial hepatectomy. For partial hepatectomy, the mice were anesthetized and a lobe of liver was surgically removed. Total cellular RNA was isolated from livers of all transgenic founders, and 5 negative control littermates as described 10 (McDonald et al. Meth. Enzymol. 152, 219 (1987)). Northern blot analysis was performed on these samples to assess the level of transgene expression. Approximately 10ug of total RNA from each animal liver was resolved by electrophoresis denaturing gels (Ogden et al. Meth. 15 Enzymol 152, 61 (1987)), then transferred to HYBOND-N nylon membrane (Amersham), and probed with ³²P dCTP-labelled pBI.1 insert DNA. Hybridization was performed overnight at 42°C in 50% Formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution, 100 µg/ml denatured 20 salmon sperm DNA and 2-4 x 10⁶ cpm of labeled probe/ml of hybridization buffer. Following hybridization, blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each, and then twice in 0.1 x SSC, 0.1% SDS at 55°C for 5-10 min each. Expression of the 25 transgene in founder and control littermates was determined following autoradiography.

The northern blot data indicate that 7 of the transgenic founders express detectable levels of the transgene mRNA (animal #'s 2,11,16,17,22,33, and 45). 30 The negative control mice and one of the founders (#28) expressed no transgene-related mRNA. Since OPG is predicted to be a secreted protein, overexpression of transgene mRNA should be a proxy for the level of systemically delivered gene product. Of the PCR and

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northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

5

EXAMPLE 4
Biological activity of OPG

10 Five of the transgenic mice (animals 2,11,16,17 and 28) and 5 control littermates (animals 1,12,15,18, and 30) were sacrificed for necropsy and pathological analysis using the following procedures: Prior to euthanasia, all animals had their
15 identification numbers verified, then were weighed, anesthetized and blood drawn. The blood was saved as both serum and whole blood for a complete serum chemistry and hematology panel. Radiography was performed just after terminal anesthesia by lethal CO₂ inhalation, and prior to the gross dissection.
20 Following this, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected included the liver, spleen, pancreas, stomach, duodenum, ileum, colon, kidney, reproductive
25 organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, jejunem, cecum, rectum, adrenals, urinary bladder, and skeletal muscle. Prior to fixation the whole organ weights were determined for the liver, stomach, kidney, adrenals,
30 spleen, and thymus. After fixation the tissues were processed into paraffin blocks, and 3 um sections were obtained. Bone tissue was decalcified using a formic acid solution, and all sections were stained with hematoxylin and eosin. In addition, staining with

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Gomori's reticulin and Masson's trichrome were performed on certain tissues. Enzyme histochemistry was performed to determine the expression of tartrate resistant acid phosphatase (TRAP), an enzyme highly expressed by
5 osteoclasts, multinucleated bone-resorbing cells of monocyte-macrophage lineage. Immunohistochemistry for BrdU and F480 monocyte-macrophage surface antigen was also performed to detect replicating cells and cells of the monocyte-macrophage lineage, respectively. To
10 detect F480 surface antigen expression, formalin fixed, paraffin embedded 4 μ m sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat
15 monoclonal anti-mouse F480 (Harlan, Indianapolis, IN). This antibody was detected by biotinylated rabbit anti-rat immunoglobulins, peroxidase conjugated strepavidin (BioGenex San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with
20 hematoxylin.

Upon gross dissection and observation of visceral tissues, no abnormalities were found in the transgene expressors or control littermates. Analysis of organ weight indicate that spleen size increased by
25 approximately 38% in the transgenic mice relative to controls. There was a slight enlargement of platelet size and increased circulating unstained cells in the transgene expressors. There was a marginal decrease in platelet levels in the transgene expressors. In
30 addition, the serum uric acid, urea nitrogen, and alkaline phosphatase levels all trended lower in the transgene expressors. The expressors were found to have increased radiodensity of the skeleton, including long bones (femurs), vertebrae, and flat bones (pelvis). The

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relative size of femurs in the expressors were not different from the control mice.

Histological analysis of stained sections of bone from the OPG expressors show severe osteopetrosis with the presence of cartilage remnants from the primary spongiosa seen within bone trabeculae in the diaphysis of the femur. A clearly defined cortex was not identifiable in the sections of femur. In normal animals, the central diaphysis is filled with bone marrow. Sections of vertebra also show osteopetrotic changes implying that the OPG-induced skeletal changes were systemic. The residual bone marrow showed predominantly myeloid elements. Megakaryocytes were present. Reticulin stains showed no evidence for reticulin deposition. Immunohistochemistry for F480, a cell surface antigen expressed by cells of monocyte-macrophage derivation in the mouse, showed the presence of F480 positive cells in the marrow spaces. Focally, flattened F480 positive cells could be seen directly adjacent to trabecular bone surfaces.

The mesenchymal cells lining the bony trabeculae were flattened and appeared inactive. Based on H&E and TRAP stains, osteoclasts were rarely found on the trabecular bone surfaces in the OPG expressors. In contrast, osteoclasts and/or chondroclasts were seen in the region of the growth plate resorbing cartilage, but their numbers may be reduced compared to controls. Also, osteoclasts were present on the cortical surface of the metaphysis where modelling activity is usually robust. The predominant difference between the expressors and controls was the profound decrease in trabecular osteoclasts, both in the vertebrae and femurs. The extent of bone accumulation was directly correlated with

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the level of OPG transgene mRNA detected by northern blotting of total liver RNA.

The spleens from the OPG expressors had an increased amount of red pulp with the expansion due to 5 increased hematopoiesis. All hematopoietic lineages are represented. F480 positive cells were present in both control and OPG expressors in the red pulp. Two of the expressors (2 and 17) had foci of extramedullary hematopoiesis within the liver and this is likely due to 10 the osteopetrotic marrow.

There were no observable abnormalities in the thymus, lymph nodes, gastrointestinal tract, pancreateo-hepatobiliary tract, respiratory tract, reproductive system, genito-urinary system, skin, nervous system, 15 heart and aorta, breast, skeletal muscle and fat.

EXAMPLE 5

Isolation of mouse and human OPG cDNA

20

A cDNA clone corresponding to the 5' end of the mouse OPG mRNA was isolated from a mouse kidney cDNA library (Clontech) by PCR amplification. The oligonucleotides were derived from the rat OPG cDNA 25 sequence and are shown below:

5'-ATCAAAGGCAGGGCATACTCCTG-3' (SEQ ID NO:13)
5'-GTTGCACTCCTGTTCACGGTCTG-3' (SEQ ID NO:14)

30 5'-CAAGACACCTTGAAGGGCCTGATG-3' (SEQ ID NO:15)
5'-TAACTTTACAGAAGAGCATCAGC-3' (SEQ ID NO:16)

5'-AGCGCGGCCGATGAACAAGTGGCTGTGCTGCG-3' (SEQ ID NO:17)
5'-AGCTCTAGAGAACAGCCCAGTGACCATTCC-3' (SEQ ID NO:18)

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The partial and full-length cDNA products obtained in this process were sequenced. The full-length product was digested with Not I and Xba I, then 5 directionally cloned into the plasmid vector pRcCMV (Invitrogen). The resulting plasmid was named pRcCMV-Mu-OPG. The nucleotide sequence of the cloned product was compared to the rat OPG cDNA sequence. Over the 1300 bp region spanning the OPG LORF, the rat and mouse 10 DNA sequences are approximately 88% identical. The mouse cDNA sequence contained a 401 aa LORF, which was compared to the rat OPG protein sequence and found to be ~94% identical without gaps. This indicates that the mouse cDNA sequence isolated encodes the murine OPG 15 protein, and that the sequence and structure has been highly conserved throughout evolution. The mouse OPG protein sequence contains an identical putative signal peptide at its N-terminus, and all 4 potential sites of N-linked glycosylation are conserved.

20 A partial human OPG cDNA was cloned from a human kidney cDNA library using the following rat-specific oligonucleotides:

25 5'-GTG AAG CTG TGC AAG AAC CTG ATG-3'
(SEQ ID NO:19)
5'-ATC AAA GGC AGG GCA TAC TTC CTG-3'
(SEQ ID NO:20)

30 This PCR product was sequenced and used to design primers for amplifying the 3' end of the human cDNA using a human OPG genomic clone in lambda as template:

5'-TCCGTAAGAACAGCCCAGTGACC-3' (SEQ ID NO:29)

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5' -CAGATCCTGAAGCTGCTCAGTTG-3' (SEQ ID NO:21)

The amplified PCR product was sequenced, and together with the 5' end sequence, was used to design 5' and 3' human-specific primers useful for amplifying the entire human OPG cDNA coding sequences:

5' -AGCGCGGCCGGGGACCAATGAACAAAGTTG-3' (SEQ ID NO:22)

5' -AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3' (SEQ ID NO:23)

10

The full-length human PCR product was sequenced, then directionally cloned into the plasmid vector pRcCMV (Invitrogen) using Not I and Xba I. The resulting plasmid was named pRcCMV-human OPG. The 15 nucleotide sequence of the cloned product was compared to the rat and mouse OPG cDNA sequences. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 78-88% identical to the human OPG cDNA. The human OPG cDNA sequence also 20 contained a 401 aa LORF, and it was compared to the rat and mouse protein sequences. The predicted human OPG protein is approximately 85% identical, and ~90% identical to the rat and mouse proteins, respectively. Sequence alignment of rat, mouse and human proteins show 25 that they have been highly conserved during evolution. The human protein is predicted to have a N-terminal signal peptide, and 5 potential sites of N-linked glycosylation, 4 of which are conserved between the rat and mouse OPG proteins.

30

The DNA and predicted amino acid sequence of mouse OPG is shown in Figure 9A and 9B (SEQ ID NO:122). The DNA and predicted amino acid sequence of human OPG is shown in Figure 9C and 9D (SEQ ID NO:124). A

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comparison of the rat, mouse and human OPG amino acid sequences is shown in Figure 9E and 9F.

Isolation of additional human OPG cDNA clones revealed the presence of a G to C base change at 5 position 103 of the DNA sequence shown in Figure 9C. This nucleotide change results in substitution of an asparagine for a lysine at position 3 of the amino acid sequence shown in Figure 9C. The remainder of the sequence in clones having this change was identical to 10 that in Figure 9C and 9D.

EXAMPLE 6
OPG three-dimensional structure modelling

15

The amino-terminal portion of OPG has homology to the extracellular portion of all known members of the TNFR superfamily (Figure 1C). The most notable motif in this region of TNFR-related genes is an ~40 amino acid, 20 cysteine-rich repeat sequence which folds into distinct structures (Banner et al. *Cell* 73, 431-445 (1993)). This motif is usually displayed in four (range 3-6) tandem repeats (see Figure 1C), and is known to be involved in ligand binding (Beutler and van Huffel 25 *Science* 264, 667-663 (1994)). Each repeat usually contains six interspaced cysteine residues, which are involved in forming three intradomain disulfide bonds, termed SS1, SS2, and SS3 (Banner et al., *ibid*). In some receptors, such as TNFR2, CD30 and CD40, some of the 30 repeat domains contain only two intrachain disulfide bonds (SS1 and SS3).

The human OPG protein sequence was aligned to a TNFR1 extracellular domain profile using methods described by Luethy, et al., *ibid*, and the results were

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graphically displayed using the PrettyPlot program from the Wisconsin Package, version 8.1 (Genetics Computer Group, Madison, WI) (Figure 10). The alignment indicates a clear conservation of cysteine residues 5 involved in formation of domains 1-4. This alignment was then used to construct a three-dimensional (3-D) model of the human OPG N-terminal domain using the known 3-D structure of the extracellular domain of p55 TNFR1 (Banner et al., *ibid*) as the template. To do this the 10 atomic coordinates of the peptide backbone and side chains of identical residues were copied from the crystal structure coordinates of TNFR1. Following this, the remaining coordinates for the insertions and different side chains were generated using the LOOK 15 program (Molecular Applications Group, Palo Alto, CA). The 3-D model was then refined by minimizing its conformational energy using LOOK.

By analogy with other TNFR family members, it is assumed that OPG binds to a ligand. For the purpose 20 of modelling the interaction of OPG with its ligand, the crystal structure of TNF- β was used to simulate a 3-D representation of an "OPG ligand". This data was graphically displayed (see Figure 11) using Molscript 25 (Kraulis, J. Appl. Cryst. **24**, 946-950, 1991). A model for the OPG/ligand complex with 3 TNF β and 3 OPG molecules was constructed where the relative positions of OPG are identical to TNFR1 in the crystal structure. This model was then used to find the residues of OPG 30 that could interact with its ligand using the following approach: The solvent accessible area of all residues in the complex and one single OPG model were calculated. The residues that have different accessibility in the complex than in the monomer are likely to interact with the ligand.

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The human and mouse OPG amino acid sequences were realigned using this information to highlight sequences comprising each of the cysteine rich domains 1-4 (Figure 12A and 12B). Each domain has individual structural characteristics which can be predicted:

Domain 1

Contains 4 cysteines involved in SS2 (C41 to C54) and SS3 (C44 to C62) disulfide bonds. Although no SS1 bond is evident based on disulfide bridges, the conserved tyrosine at position 28 is homologous to Y20 in TNFR1, which is known to be involved in interacting with H66 to aid in domain formation. OPG has a homologous histidine at position 75, suggesting OPG Y28 and H75 stack together in the native protein, as do the homologous residues in TNFR1. Therefore, both of these residues may indeed be important for biological activity, and N-terminal OPG truncations up to and beyond Y28 may have altered activity. In addition, residues E34 and K43 are predicted to interact with a bound ligand based on our 3-dimensional model.

Domain 2

Contains six cysteines and is predicted to contain SS1 (C65 to C80), SS2 (C83 to C98) and SS3 (C87 to C105) disulfide bonds. This region of OPG also contains a region stretching from P66-Q91 which aligns to the portion of TNFR1 domain 2 which forms close contacts with TNF β (see above), and may interact with an OPG ligand. In particular residues P66, H68, Y69, Y70, T71, D72, S73, H75, T76, S77, D78, E79, L81, Y82, P85, V86, K88, E89, L90, and Q91 are predicted to interact with a bound ligand based on our structural data.

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Domain 3

Contains 4 cysteines involved in SS1 (C107 to C 118) and SS3 (C124 to C142) disulfide bonds, but not an SS2 bond. Based on our structural data, residues 5 E115, L118 and K119 are predicted in to interact with an OPG ligand.

Domain 4

Contains 4 cysteines involved in SS1 (C145 to 10 C160) and SS3 (C166 to C185) disulfide bonds, but not an SS2 bond, similar to domain 3. Our structural data predict that E153 and S155 interact with an OPG ligand.

Thus, the predicted structural model for OPG 15 identifies a number of highly conserved residues which are likely to be important for its biological activity.

EXAMPLE 7

20 Production of recombinant secreted
OPG protein in mammalian cells

To determine if OPG is actually a secreted protein, mouse OPG cDNA was fused to the human IgG1 Fc 25 domain as a tag (Capon et al. *Nature* 337, 525-531 (1989)), and expressed in human 293 fibroblasts. Fc fusions were carried out using the vector pFc-A3. pFc-A3 contains the region encoding the Fc portion of human immunoglobulin IgG- γ 1 heavy chain (Ellison et al. *ibid*) 30 from the first amino acid of the hinge domain (Glu-99) to the carboxyl terminus and is flanked by a 5'-NotI fusion site and 3'-SalI and XbaI sites. The plasmid was constructed by PCR amplification of the human spleen cDNA library (Clontech). PCR reactions were in a final

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volume of 100 μ l and employed 2 units of Vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 μ M $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO₄, 0.1% Triton X-100 with 400 μ M each dNTP and 1 ng of the cDNA library

5 to be amplified together with 1 μ M of each primer.

Reactions were initiated by denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 73°C for 2 min. The 5' primer

10 5' ATAGCGGCCGCTGAGCCCCAAATCTTGTGACAAAACTCAC 3' (SEQ ID NO:24)

incorporated a NotI site immediately 5' to the first residue (Glu-99) of the hinge domain of IgG- γ 1. The 3' primer

15 5'-TCTAGAGTCGACTTATCATTACCCGGAGACAGGGAGAGGGCTCTT-3' (SEQ ID NO:25)

incorporated SalI and XbaI sites. The 717-bp PCR product was digested with NotI and SalI, isolated by electrophoresis through 1% agarose (FMC Corp.), purified by the Geneclean procedure (BIO 101, Inc.) and cloned 20 into NotI, SalI-digested pBluescript II KS vector (Stratagene). The insert in the resulting plasmid, pFc-A3, was sequenced to confirm the fidelity of the PCR reaction.

25 The cloned mouse cDNA in plasmid pRcCMV-MuOPG was amplified using the following two sets of primer pairs:

Pair 1

30 5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:26)

5'-CCTCTGCAGGCCGCTAAGCAGCTTATTTCACGGATTGAAACCTG-3' (SEQ ID NO:27)

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Pair 2

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAAG-3' (SEQ ID NO:28)

5'-CCTCTGCGGCCGCTGTTGCATTCCTTCTG-3' (SEQ ID NO:30)

5

The first pair amplifies the entire OPG LORF, and creates a NotI restriction site which is compatible with the in-frame Not I site in Fc fusion vector pFcA3. pFcA3 was prepared by engineering a NotI restriction site 5' to aspartic acid residue 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the junction between the OPG protein and the IgG Fc region. This product, when linked to the Fc portion, would encode all 401 OPG residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (Fl.Fc). The second primer pair amplifies the DNA sequences encoding the first 180 amino acid residues of OPG, which encompasses its putative ligand binding domain. As above, the 3' primer creates an artificial Not I restriction site which fuses the C-terminal truncated OPG LORF at position threonine 180 directly to the IgG1 Fc domain (CT.fc).

The amino acid sequence junction linking OPG residue 401 and aseptic acid residue 221 of the human Fc region can be modified as follows: The DNA encoding residues 216-220 of the human Fc region can be deleted as described below, or the cysteine residue corresponding to C220 of the human Fc region can be mutated to either serine or alanine. OPF-Fc fusion protein encoded by these modified vectors can be transfected into human 293 cells, or CHO cells, and recombinant OPG-Fc fusion protein purified as described below.

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Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is capable of episomal replication in 293-EBNA-1 cells.

5 The parent pCEP4, and pCEP4-F1.Fc and pCEP4-CT.Fc vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 µg/ml hygromycin to select for vector expression, and the resulting drug-
10 resistant mass cultures were grown to confluence. The cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced by
15 the drug resistant 293 cultures. In the pCEP4-F1.Fc and the pCEP4-CT.Fc conditioned media, unique bands of the predicted sizes were abundantly secreted (see Figures 13B and 13C). The full-length Fc fusion protein accumulated to a high concentration, indicating that it
20 may be stable. Both Fc fusion proteins were detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant OPG products.

The full length OPG-Fc fusion protein was purified by Protein-A column chromatography (Pierce)
25 using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira et al. (J. Biol. Chem. 262, 10-35 (1987)). The following amino acid sequence was read after 19 cycles:
30

NH₂-E T L P P K Y L H Y D P E T G H Q L L-CO₂H
(SEQ ID NO:31)

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This sequence was identical to the predicted mouse OPG amino acid sequence beginning at amino acid residue 22, suggesting that the natural mammalian leader cleavage site is between amino acid residues Q21-E22, 5 not between Y31-D32 as originally predicted. The expression experiments performed in 293-EBNA cells with pCEP4-F1.Fc and pCEP4-CT.Fc demonstrate that OPG is a secreted protein, and may act systemically to bind its ligand.

10 Procedures similar to those used to construct and express the muOPG[22-180]-Fc and muOPG[22-401]-Fc fusions were employed for additional mouse and human OPG-Fc fusion proteins.

Murine OPG cDNA encoding amino acids 1-185 15 fused to the Fc region of human IgG1 [muOPG Ct(185).Fc] was constructed as follows. Murine OPG cDNA from plasmid pRcCMV Mu Osteoprotegerin (described in Example 5) was amplified using the following primer pair in a polymerase chain reaction as described above:

20

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:32)

1333-80:

5'-CCT CTG CGG CCG CAC ACA CGT TGT CAT GTG TTG C-3'
25 (SEQ ID NO:33)

This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 63-185 (corresponding to bp 278-645) of the OPG reading frame 30 as shown in Figure 9A. The 3' primer contains a Not I restriction site which is compatible with the in-frame Not I site of the Fc fusion vector pFcA3. The product also spans a unique EcoRI restriction site located at bp 436. The amplified PCR product was purified, cleaved

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with NotI and EcoRI, and the resulting EcoRI-NotI restriction fragment was purified. The vector pCEP4 having the murine 1-401 OPG-Fc fusion insert was cleaved with EcoRI and NotI, purified, and ligated to the PCR product generated above. The resulting pCEP4-based expression vector encodes OPG residues 1-185 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-185.Fc fusion vector was transfected into 293 cells, drug selected, and conditioned media was produced as described above. The resulting secreted murine OPG 1-185.Fc fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

Murine OPG DNA encoding amino acid residues 1-194 fused to the Fc region of human IgG1 (muOPG Ct(194).Fc) was constructed as follows. Mouse OPG cDNA from plasmid pRCCMV Mu-Osteoprotegerin was amplified using the following primer pairs:

20

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:34)

1333-81:

5'-CCT CTG CGG CCG CCT TTT GCG TGG CTT CTC TGT T-3'

25 (SEQ ID NO:35)

This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 70-194 (corresponding to bp 298-672) of the OPG reading frame. 30 The 3' primer contains a Not I restriction site which is compatible with the in-frame Not I site of the Fc fusion vector pFcA3. The product also spans a unique EcoRI restriction site located at bp 436. The amplified PCR product was cloned into the murine OPG[1-401] Fc fusion

- 63 -

vector as described above. The resulting pCEP4-based expression vector encodes OPG residues 1-194 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-194.Fc fusion vector 5 was transfected into 293 cells, drug selected, and conditioned media was produced. The resulting secreted fusion product was purified by Protein-A column chromatography (Pierce), using the manufacturers recommended procedures.

10

Human OPG DNA encoding amino acids 1-401 fused to the Fc region of human IgG1 was constructed as follows. Human OPG DNA in plasmid pRccCMV-hu osteoprotegerin (described in Example 5) was amplified 15 using the following oligonucleotide primers:

1254-90:

5'CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'
(SEQ ID NO:36)

20

1254-95:

5'-CCT CTG CGG CCG CTA AGC AGC TTA TTT TTA CTG AAT GG-3'
(SEQ ID NO:37)

25

The resulting PCR product encodes the full-length human OPG protein and creates a Not I restriction site which is compatible with the in-frame Not I site Fc fusion vector FcA3. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above.

30

The resulting expression vector encodes human OPG residues 1-401 directly followed by 227 amino acid residues of the human IgG1 Fc region. Conditioned media from transfected and drug selected cells was produced and the huOPG F1.Fc fusion product was purified by

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Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acid residues 1-
5 201 fused to the Fc region of human IgG1 [huOPG
Ct(201).Fc] was constructed as follows. The cloned human
OPG cDNA from plasmid pRrCMV-hu osteoprotegerin was
amplified by PCR using the following oligonucleotide
primer pair:

10

1254-90:

5'-CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT
G-3' (SEQ ID NO:38)

1254-92:

15 5'-CCT CTG CGG CCG CCA GGG TAA CAT CTA TTC CAC-3'
(SEQ ID NO:39)

This primer pair amplifies the human OPG cDNA
region encoding amino acid residues 1-201 of the OPG
20 reading frame, and creates a Not I restriction site at
the 3' end which is compatible with the in-frame Not I
site Fc fusion vector FcA3. This product, when linked
to the Fc portion, encodes OPG residues 1-201 directly
followed by all 221 amino acid residues of the human
25 IgG1 Fc region. The PCR product was directionally
cloned into the plasmid vector pCEP4 as described above.
Conditioned media from transfected and drug selected
cells was produced, and the hu OPG Ct(201).Fc fusion
products purified by Protein-A column chromatography
30 (Pierce) using the manufacturer's recommended
procedures.

The following procedures were used to
construct and express unfused mouse and human OPG.

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A plasmid for mammalian expression of full-length murine OPG (residues 1-401) was generated by PCR amplification of the murine OPG cDNA insert from pRcCMV Mu-Osteoprotegerin and subcloned into the expression vector pDSR α (DeClerck et al. J. Biol. Chem. 266, 3893 (1991)). The following oligonucleotide primers were used:

1295-26:

10 5'-CCG AAG CTT CCA CCA TGA ACA AGT GGC TGT GCT
GC-3' (SEQ ID NO:40)

1295-27:

15 5'-CCT CTG TCG ACT ATT ATA AGC AGC TTA TTT TCA CGG
ATT G-3' (SEQ ID NO:41)

The murine OPG full length reading frame was amplified by PCR as described above. The PCR product was purified and digested with restriction endonucleases 20 Hind III and Xba I (Boehringer Mannheim, Indianapolis, IN) under the manufacturers recommended conditions, then ligated to Hind III and Xba I digested pDSR α .

Recombinant clones were detected by restriction endonuclease digestion, then sequenced to ensure no 25 mutations were produced during the PCR amplification steps.

The resulting plasmid, pDSR α -muOPG was introduced into Chinese hamster ovary (CHO) cells by calcium mediated transfection (Wigler et al. Cell 11, 30 233 (1977)). Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the murine OPG recombinant protein was monitored by western blot analysis of CHO

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cell conditioned media. High expressing cells were selected, and OPG expression was further amplified by treatment with methotrexate as described (DeClerck et al., *idid*). Conditioned media from CHO cell lines was
5 produced for further purification of recombinant secreted murine OPG protein.

A plasmid for mammalian expression of full-length human OPG (amino acids 1-401) was generated by
10 subcloning the cDNA insert in pRcCMV-hu Osteoprotegerin directly into vector pDSR α (DeClerck et al., *ibid*). The pRcCMV-OPG plasmid was digested to completion with Not I, blunt ended with Klenow, then digested to completion with Xba I. Vector DNA was digested with Hind III,
15 blunt ended with Klenow, then digested with Xba I, then ligated to the OPG insert. Recombinant plasmids were then sequenced to confirm proper orientation of the human OPG cDNA.

The resulting plasmid pDSR α -huOPG was
20 introduced into Chinese hamster ovary (CHO) cells as described above. Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the human OPG recombinant
25 protein was monitored by western blot analysis of CHO cell conditioned media. High expressing clones were selected, and OPG expression was further amplified by treatment with methotrexate. Conditioned media from CHO cell lines expressing human OPG was produced for protein
30 purification.

Expression vectors for murine OPG encoding residues 1-185 were constructed as follows. Murine OPG

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cDNA from pRcCMV-Mu OPG was amplified using the following oligonucleotide primers:

1333-82:

5' -TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:42)

1356-12:

5' -CCT CTG TCG ACT TAA CAC ACG TTG TCA TGT GTT
GC-3' (SEQ ID NO:43)

10 This primer pair amplifies the murine OPG cDNA region encoding amino acids 63-185 of the OPG reading frame (bp 278-645) and contains an artificial stop codon directly after the cysteine codon (C185), which is followed by an artificial Sal I restriction endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel purified. This fragment, which contains a open reading frame encoding residues 1-185 was then subcloned into a Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a cysteine residue located at position 185. Conditioned media from transfected and drug selected cells was produced as described above.

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1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:44)

1356-13:

5 5'-CCT CTG TCG ACT TAC TTT TGC GTG GCT TCT CTG
TT-3' (SEQ ID NO:45)

This primer pair amplifies the murine OPG cDNA region encoding amino acids 70-194 of the OPG reading frame (bp 298-672) and contains an artificial stop codon directly after the lysine codon (K194), which is followed by an artificial Sal I restriction endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel purified. This fragment, which contains an open reading frame encoding residues 1-185 was then subcloned into a Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a lysine at position 194. Conditioned media from transfected and drug selected cells was produced as described above.

30

Several mutations were generated at the 5' end of the huOPG [22-401]-Fc gene that introduce either amino acid substitutions, or deletions, of OPG between residues 22 through 32. All mutations were generated

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with the "QuickChange™ Site-Directed Mutagenesis Kit" (Stratagene, San Diego, CA) using the manufacturer's recommended conditions. Briefly, reaction mix containing huOPG [22-401]-Fc plasmid DNA template and 5 mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent *E. coli* XL1-Blue by heatshock, then plated. Plasmid DNA from 10 transformants was then sequenced to verify mutations.

The following primer pairs were used to delete residues 22-26 of the human OPG gene, resulting in the production of a huOPG [27-401]-Fc fusion protein:

15 1436-11:

5'-TGG ACC ACC CAG AAG TAC CTT CAT TAT GAC-3'
(SEQ ID NO:140)

1436-12:

20 5'-GTC ATA ATG AAG GTA CTT CTG GGT GGT CCA-3'
(SEQ ID NO:141)

25 The following primer pairs were used to delete residues 22-28 of the human OPG gene, resulting in the production of a huOPG [29-401]-Fc fusion protein:

1436-17:

5'-GGA CCA CCC AGC TTC ATT ATG ACG AAG AAA C-3'
(SEQ ID NO:142)

30

1436-18:

5'-GTT TCT TCG TCA TAA TGA AGC TGG GTG GTC C-3'
(SEQ ID NO:143)

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The following primer pairs were used to delete residues 22-31 of the human OPG gene, resulting in the production of a huOPG [32-401]-Fc fusion protein:

5

1436-27:

5'-GTG GAC CAC CCA GGA CGA AGA AAC CTC TC-3'

(SEQ ID NO:144)

10

1436-28:

5'-GAG AGG TTT CTT CGT CCT GGG TGG TCC AC-3'

(SEQ ID NO:145)

15

The following primer pairs were used to change the codon for tyrosine residue 28 to phenylalanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc Y28F fusion protein:

20

1436-29:

5'-CGT TTC CTC CAA AGT TCC TTC ATT ATG AC-3'

(SEQ ID NO:146)

25

1436-30:

5'-GTC ATA ATG AAG GAA CTT TGG AGG AAA CG-3'

(SEQ ID NO:147)

30

The following primer pairs were used to change the codon for proline residue 26 to alanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc P26A fusion protein:

1429-83:

5'-GGA AAC GTT TCC TGC AAA GTA CCT TCA TTA TG-3'

(SEQ ID NO:148)

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1429-84:

5'-CAT AAT GAA GGT ACT TTG CAG GAA ACG TTT CC-3'
(SEQ ID NO:149)

5

Each resulting muOPG [22-401]-Fc plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The 10 biological activity of each protein was assessed the in vitro osteoclast forming assay described in Example 11.

EXAMPLE 8

15 Expression of OPG in E. coli

A. Bacterial Expression Vectors

pAMG21

20 The expression plasmid pAMG21 can be derived from the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be derived from the Amgen expression vector system described in US Patent No. 4,710,473. The pCFM1656 plasmid can be derived from the described 25 pCFM836 plasmid (Patent No. 4,710,473) by: (a) destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end ligation; (b) replacing the DNA sequence between the unique AatII and ClaI restriction sites containing the 30 synthetic P_L promoter with a similar fragment obtained from pCFM636 (patent No. 4,710,473) containing the P_L promoter

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AatII

5' CTAATCCGCTCTCACCTACCAAACAATGCCCTGCAAAAATAAATTCAAT-
3' TGCAGATTAAGCGAGAGTGGATGGTTACGGGGGACGTTTATTAAGTATA-

5 -AAAAAACATACAGATAACCACATCTGCGGTGATAAAATTATCTCTGGCGGTGTTGACATAAA-
-TTTTTGATGCTATTGGTAGACGCCACTATTTAATAGAGACGCCACACTGTATTT-

-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)
-ATGGTGACCGCCACTATGACTCGTGTAGC 5' (SEQ ID NO:54)

10 Clal

and then (c) substituting the small DNA sequence between the unique *Clal* and *KpnI* restriction sites with the following oligonucleotide:

15

5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGAAATCGGTAC 3'
(SEQ ID NO:48)

20 3' TAAACTAAGATCTCCTCCTTATTGTATACCAATTGCGAACCTTAAGC 5'
(SEQ ID NO:49)

Clal

KpnI

The expression plasmid pAMG21 can then be derived from pCFM1656 by making a series of site directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the *BglII* site (plasmid bp # 180) immediately 5' to the plasmid replication promoter *PcopB* and proceeding toward the plasmid replication genes, the base pair changes are as follows:

	<u>pAMG21 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG21</u>
35	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	-	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
40	# 994	G/C	A/T
	# 1004	A/T	C/G

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	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
	# 1178	G/C	T/A
5	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
10	# 2499-2502	AGTG TCAC	GTCA CAGT
	# 2642	TCCGAGC AGGCTCG	7 bp deletion
15	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

20

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

25

	(AatII sticky end) (position #4358 in pAMG21)	5' GCGTAACGTATGCATGGTCTCC- 3' TGCACGCATGCATACTGATTACAGAGG-
30	-CCATGCGAGAGTAGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT- -GGTACGCTCTCATCCCTGACGGTCCGTAGTTATTTGCTTCCGAGTCAGCTTCTGA-	
	-GGGCCTTCGTTTATCTGTTGTCGGTAAACGCTCTCTGAGTAGGACAAATCCGC-	
35	-CCCGGAAAGCAAAATAGACAACAAACAGCCACTTGCAGAGGAGTCATCCGTGTTAGGCG-	
	-CGGGAGCGGATTTGAACGTTGCGAACGAAACGGCCGGAGGGTGGCGGGCAGGACGCCGC-	
	-GCCCTCGCCCTAAACTGCAACGCTTCGTTGCGGGCTCCCACCGCCGTCCTGCGGGCG-	
40	-CATAAAATGCCAGGCATCAAATAAGCAGAAGGCCATCCTGACGGATGCCCTTTGCGT- -GTATTTGACGGTCCGTAGTTAATCGTCTCCGGTAGGACTGCCTACCGAAAAACGCA-	
		AatII
45	-TTCTACAAACTCTTTGTTATTTCTAAATACTTCAAATATGGACGTCGTACTTAAC- -AAGATGTTGAGAAAACAAATAAAAGATTATGTAAGTTATACCTGCAGCATGAATTG-	
	-TTTAAAGTATGGCAATCAATTGCTCTGTTAAATTGCTTTAGAAATACTTGGCAGC-	
	-AAAATTCTACCCGTTAGTTAACGAGGACAATTAAACGAAATCTTATGAAACCGTCG-	
50	-GGTTTGTGATTGAGTTCAATTGCGCATGGTAAATGGAAAGTGACCGTGCCTAC- -CCAAACAACTCAAAGTAAACGCGTAACCAATTACCTTCACTGGCACGCGAATG-	

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-TACAGCCTAATATTTGAAATATCCAAGAGCTTTCCCTCGCATGCCACGCTAAC-
 -ATGTCGGATTATAAAAACCTTATAGGGTCTCGAAAAAGGAAGCTACGGGTGCGATTG-
 5 -ATTCTTTCTCTTTGTTAAATCGTTGTTGATTATTATTTGCTATATTTATTTTC-
 -TAAGAAAAAGAGAAAACCAATTAGCAACAAACTAAATAAAACGATATAAATAAAAG-
 -GATAATTATCAACTAGAGAAGGAACAATTATGGTATGTTCATACACGCATGAAAAATA-
 -CTATTAATAGTTGATCTCTCCTGTTAATTACCATACAAGTATGCGTACATTTAT-
 10 -AACTATCTATATAGTTGCTTCTGAATGTGCAAAACTAACGATTCCGAAGCCATTAT-
 -TTGATAGATATATCAACAGAAAGAGACTTACACGTTGATTGTAAGGCTTCGTAATA-
 -TAGCAGTATGAATAGGGAAACTAAACCCAGTGATAAGACCTGATGATTTGCTTCTTAA-
 15 -ATCGTCATACTTATCCCTTGATTTGGGTCACTATTCTGGACTACTAAAGCGAAGAAATT-
 -TTACATTGGAGATTTTATTCAGCATTGTTCAAATATATCCAATTATCGGTG-
 -AATGTAACCTCTAAAAAATAATGCGTAACAAAAGTTATATAAGGTTAATTAGCCAC-
 20 -AATGATTGGAGTTAGAATAATCTACTATAGGATCATATTTATTAATTAGCGTCATCAT-
 -TTACTAACCTCAATCTTATTAGATGATATCCTAGTATAAAATAATTAAATCGCAGTAGTA-
 -AATATTGCTCCATTAGGGTAATTATCCAGAATTGAAATATCAGATTTAACCATAG-
 25 -TTATAACGGAGGTAAAAAATCCCATTAAATAGGTCTTAACCTTATAGTCTAAATTGGTATC-
 -AATGAGGATAAATGTCGGAGTAATAATATTCAACATGTACCATTAGTCATATCAG-
 -TTACTCCTATTACTAGCGTCATTATTATAAGTGTACATGGTAAATCAGTATAGTC-
 -ATAAGCATTGATTAATATCATTATTGCTTCTACAGGCTTAATTTATTAATTATTCTGT-
 30 -TATTGTAACTAATTATAGTAATAACGAAGATGTCCGAAATTAAAATAATTAAAGACA-
 -AAGTGTGTCGGCATTATGTCTTCTACACCATCTCTTATCCTTACCTATTGTTGTC-
 -TTCACAGCAGCGTAAATACAGAAAGTATGGTAGAGAAATAGGAATGGATAACAAACAG-
 35 -GCAAGTTTGGGTATATATCATTAAAACGGTAATAGATTGACATTGATTCTAATAA-
 -CGTTCAAAACGCACAAATATATAGTAATTGGCATTATCTAATGTAACACTAAGATTATT-
 -ATTGGATTTCGTCACACTATTATATCGCTTGAATACAATTGTTAACATAAGTACCTG-
 40 -TAACCTAAAAACAGTGTGATAATATAGCAACTTATGTTAACAAATTGTATTATGGAC-
 -TAGGATCGTACAGGTTACGCAAGAAAATGGTTGTTATAGTCGATTAATCGATTGATT-
 -ATCCTAGCATGTCCTTACGTTCTTACCAAAATATCGCTAATTAGCTAAACTAA-
 45 -CTAGATTGTTAACTAATTAAAGGAGGAATAACATATGGTAAACGCGTTGAAATTGCA-
 -GATCTAAACAAAATTGATTAATTCCCTTATTGTATACCAATTGCGAACCTTAAGCT-

 50 -GCTCACTAGTGTGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGGAAAGAA-
 -CGAGTGTGATCACAGCTGGACGTCCCATGGTACCTCGAATGAGCTCTAGGCGCTTCTT-
 -GAAGAAGAAGAAGAAAGCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA-
 -CTTCTTCTTCTTCGGCTTCCCTCGACTCAACCGACGGTGGGACTCGTTAT-
 55 -ACTAGCATAACCCCTGGGCCTCTAAACGGGTCTGAGGGGTTTTGCTGAAAGGAGG-
 -TGATCGTATTGGGAACCCCGGAGATTGCCCAGAACTCCCCAAAAACGACTTCCTCC-

SacII

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-AACCCTCTTCACGCTTCAACGC 3' [SacII sticky end] (SEQ ID NO:50)
-TTGGCGAGAAGTGCAGAAAGTG 5' (position #5904 in pAMG21) (SEQ ID NO:46)

5

During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

10

PAMG22-His

The expression plasmid pAMG22-His can be derived from the Amgen expression vector pAMG22 by substituting the small DNA sequence between the unique NdeI (#4795) and EcoRI (#4818) restriction sites of pAMG22 with the following oligonucleotide duplex:

NdeI NheI EcoRI
 5' TATGAAACATCATCACCATCACCATGCTAGCGTTAACGCGTTGG 3'
 (SEQ ID NO: 51)

3' ACTTTGTAGTAGTGGTAGTGGTAGTACGATCGCAATTGCGAACCTTAA 5'
(SEQ ID NO: 52)

MetLysHisHisHisHisHisHisAlaSerValAsnAlaLeuGlu
(SEQ ID NO:168)

25

PAMG22

The expression plasmid pAMG22 can be derived from the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be derived from the Amgen expression vector system described in US Patent No. 4,710,473 granted December 1, 1987. The pCFM1656 plasmid can be derived from the described pCFM836 plasmid (Patent No. 4,710,473) by:
(a) destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end ligation; (b) replacing the DNA sequence between the unique AatII and ClaI restriction sites

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containing the synthetic PL promoter with a similar fragment obtained from pCFM636 (patent No. 4,710,473) containing the PL promoter

5 AatII

5' CTAATTCCGCTCTCACCTACCAAAACAATGCCCTGCAAAAAATAATTCAAT-
 3' TGCAGATTAAGGCAGAGTGGATGGTTTGTACGGGGGACGTTTTTATTTAAGTATA-
 -AAAAAACATACAGATAACCATCTGGGTGATAAAATTATCTCTGGCGGTGTTGACATAAA-
 10 -TTTTTGATGTCTATTGGTAGACGCCACTATTAAATAGAGACCGCCACAACTGTATTT-
 -TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)
 -ATGGTGACCGCCACTATGACTCGTAGC 3' (SEQ ID NO:54)

ClaI

15

and then (c) substituting the small DNA sequence between the unique *ClaI* and *KpnI* restriction sites with the following oligonucleotide:

20 5' CGATTTGATTCTAGAAGGGAGGAATAACATATGGTTAACGCGTTGAAATTCCGTAC 3'
 (SEQ ID NO:55)
 3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGAACCTTAAGC 5'
 (SEQ ID NO:56)

ClaI

KpnI

25

The expression plasmid pAMG22 can then be derived from pCFM1656 by making a series of site directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the *BglII* site (plasmid bp # 180) immediately 5' to the plasmid replication promoter *PcopB* and proceeding toward the plasmid replication genes, the base pair changes are as follows:

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	<u>PAMG22 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG22</u>
5	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	--	insert two G/C bp
10	# 679	G/C	T/A
	# 980	T/A	C/G
	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
15	# 1028	A/T	T/A
	# 1047	C/G	T/A
	# 1178	G/C	T/A
	# 1466	G/C	T/A
20	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
25	# 2499-2502	AGTG TCAC	GTCA CAGT
	# 2642	TCCGAGC AGGCTCG	7 bp deletion
30	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

The DNA sequence between the unique *AatII* (position #4364 in pCFM1656) and *SacII* (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[*AatII sticky end*] (position #4358 in pAMG22)

40 5' GCGTAACGTATGCATGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAA-
 3' TGCACCGATTGCATACGTACCGAGGGTACGCTCTCATCCCTGACGGTCCGTAGTT-
 -ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTCGTTTATCTGTTGTTGTCGGTG-
 -TATTTGCTTCCGAGTCAGCTTCTGACCCGGAAAGCAAAATAGACAACAAACAGCCAC-

45

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-AACGCTCTCCTGAGTAGGACAAATCCGCCGGAGCGGGATTGAACGTTGCGAAGCAACGG-
-TTGCAGAGGACTCATCCTGTTAGGCGCCCTGCCCTAAACTGCAACGCTCGTTGCC-
-CCCGAGGGTGGCGGGCAGGACGCCGCCATAAAACTGCCAGGCATCAAATTAAAGCAGAAG-
5 -GGGCCTCCCACCGCCCGCTGCCGTACGGTACGGTCCGTAGTTAACGCTTC-
-GCCATCCTGACGGATGGCCTTTGCGTTCTACAAACTCTTTGTTATTTCTAAAT-
-CGGTAGGACTGCCTACCGAAAAACGAAAGATGTTGAGAAAACAATAAAAGATTTA-
10 AatII
-ACATTCAAATATGGACGTCTCATAATTAAATTCATTTGACAAATGCTAAATTC-
-TGTAAGTTATACTGCAGAGTATTAATTTAAGTAAACTGTTACGATTTAAG-
15 -TTGATTAATATTCTCAATTGTGAGCGCTCACAAATTATCGATTGATTCTAGATTGTT-
-AACTAATTATAAGAGTTAACACTCGCGAGTGTAAATAGCTAAACTAAGATCTAAACTCA-
-TAACTAATTAAAGGAGGAATAACATATGGTTAACCGGTTGGAAATCGAGCTCACTAGTGT-
-ATTGATTAATTCCCTCCTTATTGTATACCAATTGCGAACCTTAAGCTCGAGTGATCACA-
20 SacII
-CGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGAAAGAAGAAGAAGAAGAA-
-GCTGGACGTCCCAGGTACCTCGAAATGAGCTCCTAGGCGCCTTCTTCTTCTTCTTCTT-
-25 -GAAAGCCCAGAGGAAGCTGAGTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC-
-CTTCGGGCTTCCTCGACTCAACCGACGACGGTGGCGACTCGTTATTGATCGTATTGG-
-CCTTGGGCTCTAAACGGGCTTGGGGTTTTGCTGAAAGGAGGAACCGCTTCA-
-GGAACCCGGAGATTGCCAGAACTCCCCAAAAACGACTTCCCTGGCGAGAAGT-
30 -CGCTCTCACCGC 3' (SEQ ID NO:58)
-GCGAGAAGTG 5' (SEQ ID NO:57)

```

[SacII sticky end] (position #5024 in pAMG22)

35 During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

40 B. Human OPG Met [32-401]

In the example, the expression vector used was pAMG21, a derivative of pCFM1656 (ATCC accession no. 69576) which contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter. 45 (See U.S. Patent No. 5,169,318 for description of the lux expression system). The host cell used was GM120

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(ATCC accession no. 55764). This host has the lacIQ promoter and lacI gene integrated into a second site in the host chromosome of a prototrophic *E. coli* K12 host. Other commonly used *E. coli* expression vectors and host 5 cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human OPG polypeptide was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as 10 a template plasmid pRcCMV-Hu OPG DNA containing the human OPG cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for 15 30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22 were 20 phosphorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and KpnI sticky ends. The phosphorylated linker duplex 25 formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and KpnI cohesive ends (see Figure 14A) and the KpnI and BamHI digested and purified PCR product generated using oligo primers #1257-20 and #1257-19 (see above) was directionally inserted between two sites of 30 the plasmid vector pAMG21, namely the NdeI site and BamHI site, using standard recombinant DNA methodology (see Figure 14A and sequences below). The synthetic linker utilized *E. coli* codons and provided for a N-terminal methionine.

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Two clones were selected and plasmid DNA isolated, and the human OPG insert was subsequently DNA sequence confirmed. The resulting pAMG21 plasmid containing amino acids 32-401 of the human OPG polypeptide immediately preceded in frame by a methionine is referred to as pAMG21-huOPG met[32-401] or pAMG21-huOPG met[32-401].

- 5 Oligo#1257-19
10 5'-TACGCACTGGATCCTTATAAGCAGCTTATTCTACTGATTGGAC-3'
 (SEQ ID NO:59)
- 15 Oligo#1257-20
 5'-GTCCTCCTGGTACCTACCTAAAACAAAC-3' (SEQ ID NO:60)
- 20 Oligo#1257-21
 5'-TATGGATGAAGAAACTTCTCATCAGCTGCTGTGTGATAAATGTCC
 GCCGGGTAC -3' (SEQ ID NO:61)
- 25 Oligo#1257-22
 5'-CCGGCGGACATTTATCACACACAGCAGCTGATGAGAAGTTCTTCATCCA-3'
 (SEQ ID NO:47)

25 Cultures of pAMG21-huOPG met[32-401] in E.
 coli GM120 in 2XYT media containing 20 µg/ml kanamycin
 were incubated at 30°C prior to induction. Induction of
 huOPG met[32-401] gene product expression from the luxPR
 promoter was achieved following the addition of the
 synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine
30 lactone to the culture media to a final concentration of
 30 ng/ml and cultures were incubated at either 30°C or
 37°C for a further 6 hours. After 6 hours, the
 bacterial cultures were examined by microscopy for the
 presence of inclusion bodies and were then pelleted by

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centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant huOPG met[32-401] gene product was produced insolubly in *E. coli*. Some bacterial pellets were resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemlli sample buffer to 1X final, and β -mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE. A substantially more intense coomassie stained band of approximately 42kDa was observed on a SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture (Figure 14B). The expected gene product would be 370 amino acids in length and have an expected molecular weight of about 42.2 kDa. Following induction at 37°C for 6 hours, an additional culture was pelleted and either processed for isolation of inclusion bodies (see below) or processed by microfluidizing. The pellet processed for microfluidizing was resuspended in 25mM Tris-HCl/pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and collected. An aliquot was removed of the collected sample (microfluidized total lysate), and the remainder was pelleted at 20,000 x g for 20 minutes. The supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea solution (microfluidized insoluble fraction). To an aliquot of either the total soluble, or insoluble fraction was added to an equal volume of 2X Laemalli sample buffer and β -mercaptoethanol to 5% final concentration. The samples were then analyzed by SDS-PAGE. A significant amount of recombinant huOPG met[32-401] gene product appeared to be found in the

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insoluble fraction. To purify the recombinant protein inclusion bodies were purified as follows: Bacterial cells were separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped
5 with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water.
This suspension was transferred to a stainless steel cup cooled in ice and subjected to sonic disruption using a
10 Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts). The sonicated cell suspension was centrifuged in a Beckman Optima TLX ultracentrifuge equipped with a TLA 100.3 rotor at 195,000 x g for 5 to 10 minutes at 23°C. The
15 supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle. The pellets were collected by scraping with a micro spatula and transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M
20 sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended. The volume was increased to 19.5 ml by the addition of Percoll solution, mixed, and distributed into 3 Beckman Quick-Seal tubes (13 x 32 mm). Tubes were sealed
25 according to manufacturers instructions. The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600 x g), 30 minutes. The tubes were examined for the appropriate banding pattern. To recover the refractile bodies, gradient fractions were recovered and pooled, then diluted with water. The inclusion bodies
30 were pelleted by centrifugation, and the protein concentration estimated following SDS-PAGE.

An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemlli sample

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buffer with 5% β -mercaptoethanol and resolved on a SDS-PAGE gel and the isolated inclusion bodies provide a highly purified recombinant huOPG[32-401] gene product. The major ~42 kDa band observed after resolving

5 inclusion bodies on a SDS-polyacrylamide gel was excised from a separate gel and the N-terminal amino acid sequence determined essentially as described (Matsudaira et al. J. Biol. Chem. 262, 10-35 (1987)). The following sequence was determined after 19 cycles:

10

NH₂ -MDEETSHQLLCDKC α PGTY-COOH (SEQ ID NO:62)

This sequence was found to be identical to the first 19 amino acids encoded by the pAMG21 Hu-OPG met[32-401] expression vector, produced by a methionine residue provided by the bacterial expression vector.

C. Human OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. Isolated plasmid DNA of pAMG21-huOPG met[32-401] (see Section B) was cleaved with KpnI and BamHI restriction endonucleases and the resulting fragments were resolved on an agarose gel. The B fragment (~1064 bp fragment) was isolated from the gel using standard methodology. Synthetic oligonucleotides (oligos) #1267-06 and #1267-07 were phosphorylated individually and allowed to form an oligo linker duplex, which contained NdeI and KpnI cohesive ends, using methods described in Section 3. The synthetic linker duplex utilized *E. coli* codons and provided for an N-terminal methionine. The phosphorylated oligo linker containing NdeI and KpnI

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cohesive ends and the isolated ~1064 bp fragment of pAMG21-huOP met[32-401] digested with KpnI and BamHI restriction endonucleases were directionally inserted between the NdeI and BamHI sites of pAMG21 using
5 standard recombinant DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of
10 the huOPG-met[22-401] gene.

Oligo #1267-06

5'-TAT GGA AAC TTT TCC TCC AAA ATA TCT TCA TTA TGA TGA
AGA AAC TTC TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC GGG
15 TAC-3' (SEQ ID NO:63)

Oligo #1267-07

5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAG AAG TTT
CTT CAT CAT AAT GAA GAT ATT TTG GAG GAA AAG TTT CCA-3'
20 (SEQ ID NO:64)

Cultures of pAMG21-huOPG-met[22-401] in E. coli host 393 were placed in 2XYT media containing 20 µg/ml kanamycin and were incubated at 30°C prior to
25 induction. Induction of recombinant gene product expression from the luxPR promoter of vector pAMG21 was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and incubation at either 30°C or 37°C for a further 6 hours. After 6 hours, bacterial cultures were pelleted by centrifugation (=30°C I+6 or 37°C I+6). Bacterial cultures were also either pelleted just prior to induction (=30°C PreI) or alternatively no autoinducer
30

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was added to a separate culture which was allowed to incubate at 30°C for a further 6 hours to give an uninduced (UI) culture (=30°C UI). Bacterial pellets of either 30°C PreI, 30°C UI, 30°C I+6, or 37°C I+6 cultures were resuspended, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Section B. Polyacrylamide gels were either stained with coomassie blue and/or Western transferred to nitrocellulose and immunoprobed with rabbit anti-mu OPG-Fc polyclonal antibody as described in Example 10. The level of gene product following induction compared to either an uninduced (30°C UI) or pre-induction (30°C PreI) sample.

15 D. Murine OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of the murine (mu) OPG (OPG) polypeptide was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1257-16 and #1257-15 as primers, plasmid pRcCMV-Mu OPG DNA as a template and thermocycling conditions as described in Section B. The PCR product was purified and cleaved with KpnI and BamHI restriction endonucleases as described in Section B. Synthetic oligos #1260-61 and #1260-82 were phosphorylated individually and allowed to form an oligo linker duplex with NdeI and KpnI cohesive ends using methods described in Section B. The synthetic linker duplex utilized *E. coli* codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1260-61 and #1260-82 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product generated using oligo

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primers #1257-16 and #1257-15 were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into *E. coli* host 393 by electroporation 5 utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG met[22-401] gene.

10 Expression of recombinant muOPG met[22-401] polypeptide from cultures of 393 cells harboring plasmid pAMG21-MuOPG met[22-401] following induction was determined using methods described in Section C.

15 Oligo #1257-15
5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTC ACG GAT TGA AC-3' (SEQ ID NO:65)

20 Oligo #1257-16
5'-GTG CTC CTG GTA CCT ACC TAA AAC AGC ACT GCA CAG TG-3' (SEQ ID NO:66)

Oligo #1260-61
5'-TAT GGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC 25 GGG TAC-3' (SEQ ID NO:67)

Oligo #1260-82
5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CCA- 30 3' (SEQ ID NO:68)

E. Murine OPG met[32-401]

A DNA sequence coding for an N-terminal methionine and amino acids 32 through 401 of murine OPG.

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was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. To accomplish this, Synthetic oligos #1267-08 and #1267-09 were phosphorylated individually and allowed to form
5 an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1267-08 and #1267-09 containing NdeI and KpnI cohesive
10 ends, and the KpnI and BamHI digested and purified PCR product described earlier (see Section D), was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by
15 electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met [32-401] gene.

Expression of recombinant muOPG-met [32-401]
20 polypeptide from cultures of 393 cells harboring the pAMG21 recombinant plasmid following induction was determined using methods described in Section C.

Oligo #1267-08
25 5'-TAT GGA CCC AGA AAC TGG TCA TCA GCT GCT GTG TGA
TAA ATG TGC TCC GGG TAC-3' (SEQ ID NO:69)

Oligo #1267-09
30 5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CTG GGT CCA-3' (SEQ ID NO:70)

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E. Murine OPG met-lys[22-401]

A DNA sequence coding for an N-terminal methionine followed by a lysine residue and amino acids 22 through 401 of murine OPG was placed under control of 5 the lux PR promoter in prokaryotic expression vector pAMG21 as follows. Synthetic oligos #1282-95 and #1282-96 were phosphorylated individually and allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized *E. coli* codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos 10 #1282-95 and #1282-96 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product described in Section D was directionally 15 inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing 20 was performed to verify the DNA sequence of the MuOPG--Met-Lys[22-401] gene.

Expression of recombinant MuOPG Met-Lys[22-401] polypeptide from transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was 25 determined using methods described in Section C.

Oligo #1282-95

5'-TAT GAA AGA AAC TCT GCC TCC AAA ATA CCT GCA TTA
CGA TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC
30 TCC GGG TAC-3' (SEQ ID NO:71)

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Oligo #1282-96

5' -CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT
TCA-3' (SEQ ID NO:72)

5

G. Murine OPG met-lys-(his)7[22-401]

A DNA sequence coding for N-terminal residues Met-Lys-His-His-His-His-His (=MKH) followed by amino acids 22 through 401 of Murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-50 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases and purified. The NdeI and BamHI digested and purified PCR product generated using oligo primers #1300-50 and #1257-15 was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing performed to verify the DNA sequence of the muOPG-MKH[22-401] gene.

Expression of recombinant MuOPG-MKH[22-401] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

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Oligo #1300-50

5'-GTT CTC CTC ATA TGA AAC ATC ATC ACC ATC ACC ATC
ATG AAA CTC TGC CTC CAA AAT ACC TGC ATT ACG AT-3' (SEQ
ID NO:73)

5

Oligo #1257-15

(see Section D)

H. Murine OPG met-lys[22-401](his)7

10 A DNA sequence coding for a N-terminal met-
lys, amino acids 22 through 401 murine OPG, and seven
histidine residues following amino acid 401 (=muOPG
MK[22-401]-H7), was placed under control of the lux PR
promoter in prokaryotic expression vector pAMG21 as
15 follows. PCR was performed using oligonucleotides
#1300-49 and #1300-51 as primers and pAMG21-muOPG
met[22-401] DNA as template. Thermocycling conditions
were as described in Section B. The resulting PCR
sample was resolved on an agarose gel, the PCR product
20 was excised, purified, restricted with NdeI and BamHI
restriction endonucleases, and purified. The NdeI and
BamHI digested and purified PCR product was
directionally inserted between the NdeI and BamHI sites
in pAMG21 using standard methodology. The ligation was
25 transformed into E. coli host 393 by electroporation
utilizing the manufacturer's protocol. Clones were
selected, plasmid DNA was isolated, and DNA sequencing
was performed to verify the DNA sequence of the muOPG
MK[22-401]-H7 gene.

30 Expression of the recombinant muOPG
MK-[22-401]-H7 polypeptide from a transformed 393 cells
harboring the recombinant pAMG21 plasmid following
induction was determined using methods described in
Section C.

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Oligo #1300-49

5' -GTT CTC CTC ATA TGA AAG AAA CTC TGC CTC CAA AAT
ACC TGC A-3' (SEQ ID NO:74)

5

Oligo #1300-51

5' -TAC GCA CTG GAT CCT TAA TGA TGG TGA TGG TGA TGA
TGT AAG CAG CTT ATT TTC ACG GAT TGA ACC TGA TTC CCT A-3'
(SEQ ID NO:75)

10

I. Murine OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-74 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[27-401] gene.

Expression of recombinant muOPG-met[27-401] polypeptide from a transfected 393 culture harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

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Oligo#1309-74

5' -GTT CTC CTC ATA TGA AAT ACC TGC ATT ACG ATC CGG
AAA CTG GTC AT-3' (SEQ ID NO:76)

5

Oligo#1257-15

(See Section D)

J. Human OPG-met[27-401]

10 A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of human OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-75 and #1309-
15 76 as primers and plasmid pAMG21-huOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with AseI and BamHI restriction endonucleases, and purified. The AseI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into *E. coli* host 393 by
20 electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[27-401] gene.

25

30 Expression of the recombinant huOPG-met[27-401] polypeptide following induction of from transfected 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

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Oligo #1309-75

5'-GTT CTC CTA TTA ATG AAA TAT CTT CAT TAT GAT GAA
GAA ACT T-3' (SEQ ID NO:77)

5

Oligo #1309-76

5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTT ACT
GAT T-3' (SEQ ID NO:78)

10 K. Murine OPG met[22-180]

A DNA sequence coding for a N-terminal methionine and amino acids 22 through 180 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR 15 was performed with oligonucleotides #1309-72 and #1309-73 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, 20 restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation was 25 transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[22-180] gene.

30 Expression of recombinant muOPG-met[22-180] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

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Oligo #1309-72

5'-GTT CTC CTC ATA TGG AAA CTC TGC CTC CAA AAT ACC
TGC A-3' (SEQ ID NO:79)

5 Oligo #1309-73

5'-TAC GCA CTG GAT CCT TAT GTT GCA TTT CCT TTC TGA
ATT AGC A-3' (SEQ ID NO:80)

L. Murine OPG met[27-180]

10 A DNA sequence coding for a N-terminal methionine and amino acids 27 through 180 of murine OPG was placed under the control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-74 (see
15 Section I) and #1309-73 (see Section K) as primers and plasmid pAMG21-muOPG met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product excised, purified, restricted with NdeI
20 and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393
25 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG met[27-180] gene.

30 Expression of recombinant muOPG met[27-180] polypeptide from cultures of transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

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M. Murine OPG met[22-189] and met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 189, or 22 through 194 of murine OPG was placed under control of
5 the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1337-92 and #1337-93 (=muOPG-189 linker) or #1333-57 and #1333-58 (=muOPG-194 linker) were phosphorylated individually and allowed to form an
10 oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-muOPG-met[22-401] was cleaved with KpnI and BspEI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~413 bp B fragment was
15 isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1337-92 and #1337-93 (muOPG-189 linker) or oligos #1333-57 and #1333-58 (muOPG-194 linker) containing BspEI and BamHI cohesive ends, and the
20 isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of pAMG21-muOPG met[22-401] using standard methodology. Each ligation mixture was
25 transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the muOPG-met[22-189] or muOPG-met[22-194] genes.
30 Expression of recombinant muOPG-met[22-189] and muOPG-met[22-194] polypeptides from recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

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Oligo #1337-92

5'-CCG GAA ACA GAT AAT GAG-3' (SEQ ID NO:81)

Oligo #1337-93

5'-GAT CCT CAT TAT CTG TTT-3' (SEQ ID NO:82)

Oligo #1333-57

5'-CCG GAA ACA GAG AAG CCA CGC AAA AGT AAG-3'
(SEQ ID NO:83)

10

Oligo #1333-58

5'-GAT CCT TAC TTT TGC GTG GCT TCT CTG TTT-3'
(SEQ ID NO:84)

15 N. Murine OPG met[27-189] and met[27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 189, or 27 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers either "muOPG-189 linker" or "muOPG-194 linker" (see Section M) containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-muOPG-met[27-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the muOPG met[27-189] or muOPG met[27-194] genes.

Expression of recombinant muOPG met[27-189] and muOPG met[27-194] following induction of 393 cells

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harboring recombinant pAMG21 plasmids was determined using methods described in Section C.

O. Human OPG met[22-185], met[22-189], met[22-194]

5 A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 185, 22 through 189, or 22 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as
10 follows. The pair of synthetic oligonucleotides #1331-87 and #1331-88 (=huOPG-185 linker), #1331-89 and #1331-90 (=huOPG-189 linker), or #1331-91 & #1331-92 (=huOPG-194 linker) were phosphorylated individually and each allowed to form an oligo linker duplex pair using
15 methods described in Section B. Purified plasmid DNA of pAMG21-huOPG-met[27-401] was restricted with KpnI and NdeI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~407 bp B fragment was isolated using standard recombinant DNA methodology.
20 The phosphorylated oligo linker duplexes formed between either oligos #1331-87 and #1331-88 (huOPG-185 linker), oligos #1331-89 and #1331-90 (huOPG-189 linker), or oligos #1331-91 and #1331-92 (huOPG-194 linker) (each linker contains NdeI and BamHI cohesive
25 ends), and the isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[22-401] using standard methodology.
30 Each ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of

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either the huOPG-met[22-185], huOPG-met[22-189], or huOPG-met[22-194] genes.

5 Expression of recombinant huOPG-met[22-185], huOPG-met[22-189] or huOPG-met[22-194] in transformed 393 cells harboring recombinant pAMG21 plasmids following induction was determined using methods described in Section C.

Oligo #1331-87

10 5'-TAT GTT AAT GAG-3' (SEQ ID NO:85)

Oligo #1331-88

5'-GAT CCT CAT TAA CA-3' (SEQ ID NO:86)

15 Oligo #1331-89

5'-TAT GTT CCG GAA ACA GTT AAG-3' (SEQ ID NO:87)

Oligo #1331-90

5'-GAT CCT TAA CTG TTT CCG GAA CA-3' (SEQ ID NO:88)

20

Oligo #1331-91

5'-TAT GTT CCG GAA ACA GTG AAT CAA CTC AAA AAT
AAG-3' (SEQ ID NO:89)

25

Oligo #1331-92

5'-GAT CCT TAT TTT TGA GTT GAT TCA CTG TTT CCG GAA
CA-3' (SEQ ID NO:90)

30

P. Human OPG met[27-185], met[27-189], met[27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 185, 27 through 189, or 27 through 194 of the human OPG polypeptide was placed under control of the lux PR

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promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers "huOPG-185 linker", "huOPG-189 linker", or "huOPG-194 linker" (See Section O) each containing NdeI and BamHI cohesive ends, 5 and the isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases (See Section O) were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[27-401] (See Section J) 10 using standard methodology. Each ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA isolated, and DNA sequencing performed to verify the DNA sequence of either the 15 huOPG-met[27-185], huOPG-met[27-189], or huOPG-met[27-194] genes.

Expression of recombinant huOPG-met[27-185], huOPG-met[27-189], and huOPG-met[27-194] from recombinant pAMG21 plasmids transformed into 393 cells 20 was determined using methods described in Section C.

O. Murine OPG met[27-401] (P33E, G36S, A45P)

A DNA sequence coding for an N-terminal 25 methionine and amino acids 27 through 48 of human OPG followed by amino acid residues 49 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Purified plasmid DNA of pAMG21-huOPG-met[27-401] (See 30 Section J) was cleaved with AatII and KpnI restriction endonucleases and a ~1075 bp B fragment isolated from an agarose gel using standard recombinant DNA methodology. Additionally, plasmid pAMG21-muOPG-met[22-401] DNA (See Section D) was digested with KpnI and BamHI restriction

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endonucleases and the ~1064 bp B fragment isolated as described above. The isolated ~1075 bp pAMG21-huOPG-met[27-401] restriction fragment containing AatII & Kpnl cohesive ends (see above), the ~1064 bp pAMG21-muOPG-met[22-401] restriction fragment containing Kpnl and BamHI sticky ends and a ~5043 bp restriction fragment containing AatII and BamHI cohesive ends and corresponding to the nucleic acid sequence of pAMG21 between AatII & BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, and the presence of the recombinant insert in the plasmid verified using standard DNA methodology.

15 muOPG-27-401 (P33E, G36S, A45P) gene. Amino acid changes in muOPG from proline-33 to glutamic acid-33, glycine-36 to serine-36, and alanine-45 to proline-45, result from replacement of muOPG residues 27 through 48 with huOPG residues 27 through 48.

20 Expression of recombinant muOPG-met[27-401] (P33E, G36S, A45P) from transformed 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

25

R. Murine OPG met-lys-(his)₇-ala-ser-(asp)₄-lys[22-401]
(A45T)

A DNA sequence coding for an N-terminal His tag and enterokinase recognition sequence which is (NH₂ 30 to COOH terminus): Met-Lys-His-His-His-His-His-Ala-Ser-Asp-Asp-Asp-Lys (=HEK), followed by amino acids 22 through 401 of the murine OPG polypeptide was placed under control of the lac repressor regulated Ps4 promoter as follows. pAMG22-His (See Section A) was

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digested with NheI and BamHI restriction endonucleases, and the large fragment (the A fragment) isolated from an agarose gel using standard recombinant DNA methodology. Oligonucleotides #1282-91 and #1282-92 were
5 phosphorylated individually and allowed to form an oligo linker duplex using methods previously described (See Section B). The phosphorylated linker duplex formed between oligos #1282-91 and #1282-92 containing NheI and KpnI cohesive ends, the KpnI and BamHI digested and
10 purified PCR product described (see Section D), and the A fragment of vector pAMG22-His digested with NheI and BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into *E. coli* host GM120 by electroporation utilizing the
15 manufacturer's protocol. Clones were selected, plasmid DNA isolated and DNA sequencing performed to verify the DNA sequence of the muOPG-HEK[22-401] gene. DNA sequencing revealed a spurious mutation in the natural muOPG sequence that resulted in a single amino acid
20 change of Alanine-45 of muOPG polypeptide to a Threonine.

Expression of recombinant muOPG-HEK[22-401] (A45T) from GM120 cells harboring the recombinant pAMG21 plasmid was determined using methods similar to those
25 described in Section C, except instead of addition of the synthetic autoinducer, IPTG was added to 0.4 mM final to achieve induction.

Oligo #1282-91

30 5'-CTA GCG ACG ACG ACG ACA AAG AAA CTC TGC CTC CAA
AAT ACC TGC ATT ACG ATC CGG AAA CTG GTC ATC AGC TGC TGT
GTG ATA AAT GTG CTC CGG GTA C-3' (SEQ ID NO:91)

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Oligo #1282-92

5' -CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT
5 TGT CGT CGT CGT CG-3' (SEQ ID NO:92)

S. Human OPG met-arg-gly-ser-(his)₆[22-401]

Eight oligonucleotides (1338-09 to 1338-16 shown below) were designed to produce a 175 base 10 fragment as overlapping, double stranded DNA. The oligos were annealed, ligated, and the 5' and 3' oligos were used as PCR primers to produce large quantities of the 175 base fragment. The final PCR gene products were digested with restriction endonucleases Clal and KpnI to 15 yield a fragment which replaces the N-terminal 28 codons of human OPG. The Clal and KpnI digested PCR product was inserted into pAMG21-huOPG [27-401] which had also been cleaved with Clal and KpnI. Ligated DNA was transformed into competent host cells of E. coli strain 20 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were 25 analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Expression of huOPG Met-Arg-Gly-Ser-(His)₆ [22-401] resulting in the formation of large inclusion bodies and the protein was localized to the 30 insoluble (pellet) fraction.

1338-09

ACA AAC ACA ATC GAT TTG ATA CTA GA (SEQ ID NO:93)

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1338-10

TTT GTT TTA ACT AAT TAA AGG AGG AAT AAA ATA TGA GAG GAT CGC ATC AC
(SEQ ID NO:94)

5 1338-11

CAT CAC CAT CAC GAA ACC TTC CCG CCG AAA TAC CTG CAC TAC GAC GAA GA
(SEQ ID NO:95)

1338-12

10 AAC CTC CCA CCA GCT GCT GTG CGA CAA ATG CCC GCC GGG TAC CCA AAC A
(SEQ ID NO:96)

1338-13

TGT TTG GGT ACC CGG CGG GCA TTT GT (SEQ ID NO:97)

15

1338-14

CGC ACA GCA GCT GGT GGG AGG TTT CTT CGT CGT AGT GCA GGT ATT TCG GC
(SEQ ID NO:98)

20 1338-15

GGG AAG GTT TCG TGA TGG TGA TGG TGA TGC GAT CCT CTC ATA TTT TAT T
(SEQ ID NO:99)

1338-16

25 CCT CCT TTA ATT AGT TAA AAC AAA TCT AGT ATC AAA TCG ATT GTG TTT GT
(SEQ ID NO:100)

I. Human OPG met-lys[22-401] and met-(lys)3[22-401]

To construct the met-lys and met-(lys)3

30 versions of human OPG[22-401], overlapping oligonucleotides were designed to add the appropriate number of lysine residues. The two oligos for each construct were designed to overlap, allowing two rounds of PCR to produce the final product. The template for

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the first PCR reaction was a plasmid DNA preparation containing the human OPG 22-401 gene. The first PCR added the lysine residue(s). The second PCR used the product of the first round and added sequence back to
5 the first restriction site, Clal.

The final PCR gene products were digested with restriction endonucleases Clal and KpnI, which replace the N-terminal 28 codons of hu OPG, and then ligated into plasmid pAMG21-hu OPG [27-401] which had been also
10 digested with the two restriction endonucleases.

Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide
15 sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Neither
20 construct had a detectable level of protein expression and inclusion bodies were not visible. The DNA sequences were confirmed by DNA sequencing.

Oligonucleotide primers to prepare Met-Lys huOPG[22-
25 401]:

1338-17

ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT
AAA GGA GGA ATA AAA TG (SEQ ID NO:101)

30 1338-18

CTA ATT AAA GGA GGA ATA AAA TGA AAG AAA CTT TTC CTC CAA
AAT ATC (SEQ ID NO:102)

1338-20

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TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID NO:103)

Oligonucleotide primers to prepare Met-(Lys)₃-huOPG(22-

5 401):

1338-17

ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT AAA GGA GGA ATA AAA TG (SEQ ID NO:104)

10 1338-19

CTA ATT AAA GGA GGA ATA AAA TGA AAA AAA AAG AAA CTT TTC CTC CAA AAT ATC (SEQ ID NO:105)

1338-20

15 TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID NO:106)

U. Human and Murine OPG [22-401]/Fc Fusions

Four OPG-Fc fusions were constructed where the 20 Fc region of human IgG1 was fused at the N-terminus of either human or murine Osteoprotegerin amino acids 22 to 401 (referred to as Fc/OPG [22-401]) or at the C-terminus (referred to as OPG[22-401]/Fc). Fc fusions were constructed using the fusion vector pFc-A3 described in Example 7.

All fusion genes were constructed using standard PCR technology. Template for PCR reactions were plasmid preparations containing the target genes. Overlapping oligos were designed to combine the 30 C-terminal portion of one gene with the N terminal portion of the other gene. This process allows fusing the two genes together in the correct reading frame after the appropriate PCR reactions have been performed. Initially one "fusion" oligo for each gene was put into

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a PCR reaction with a universal primer for the vector carrying the target gene. The complimentary "fusion" oligo was used with a universal primer to PCR the other gene. At the end of this first PCR reaction, two
5 separate products were obtained, with each individual gene having the fusion site present, creating enough overlap to drive the second round of PCR and create the desired fusion. In the second round of PCR, the first
10 two PCR products were combined along with universal primers and via the overlapping regions, the full length fusion DNA sequence was produced.

The final PCR gene products were digested with restriction endonucleases XbaI and BamHI, and then
15 ligated into the vector pAMG21 having been also digested with the two restriction endonucleases. Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce
20 the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate, sonic pellet, and supernatant were analyzed for expression of the fusion by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody.
25

Fc/huOPG [22-401]

Expression of the Fc/hu OPG [22-401] fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The cells have very large inclusion
30 bodies, and the majority of the product is in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

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1318-48

CAG CCC GGG TAA AAT GGA AAC GTT TCC TCC AAA ATA TCT TCA
TT (SEQ ID NO:107)

5 1318-49

CGT TTC CAT TTT ACC CGG GCT GAG CGA GAG GCT CTT CTG CGT
GT (SEQ ID NO:108)

Fc/muOPG [22-4011]

10 Expression of the fusion peptide was detected
on a Coomassie stained gel and on a Western blot. The
cells have very large inclusion bodies, and the majority
of the product is in the insoluble (pellet) fraction.
The following primers were used to construct this OPG-Fc
15 fusion:

1318-50

CGC TCA GCC CGG GTA AAA TGG AAA CGT TGC CTC CAA AAT ACC
TGC (SEQ ID NO:109)

20

1318-51

CCA TTT TAC CCG GGC TGA GCG AGA GGC TCT TCT GCG TGT
(SEQ ID NO:110)

25 muOPG [22-4011]/Fc

Expression of the fusion peptide was detected
on a Coomassie stained gel and on a Western blot. The
amount of recombinant product was less than the OPG
fusion proteins having the Fc region in the N terminal
30 position. Obvious inclusion bodies were not detected.
Most of the product appeared to be in the insoluble
(pellet) fraction. The following primers were used to
construct this OPG-Fc fusion:

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1318-54

GAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC
(SEQ ID NO:111)

5 1318-55

CAG CTG CAG CTA AGC AGC TTA TTT TCA CGG ATT G
(SEQ ID NO:112)

10 huOPG [22-401]-Fc

Expression of the fusion peptide was not detected on a Coomassie stained gel, although a faint Western positive signal was present. Obvious inclusion bodies were not detected. The following primers were 15 used to prepare this OPG-Fc fusion:

1318-52

AAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC
(SEQ ID NO:113)

20

1318-53

CAG CTG CAG CTA AGC AGC TTA TTT TTA CTG ATT GG
(SEQ ID NO:114)

25 V. Human OPG met[22-401]-Fc fusion (P25A)

This construct combines a proline to alanine amino acid change at position 25 (P25A) with the huOPG met[22-401]-Fc fusion. The plasmid was digested with restriction endonucleases *Cla*I and *Kpn*I, which removes 30 the N-terminal 28 codons of the gene, and the resulting small (less than 200 base pair) fragment was gel purified. This fragment containing the proline to alanine change was then ligated into plasmid pAMG21-huOPG [22-401]-Fc fusion which had been digested with

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the two restriction endonucleases. The ligated DNA was transformed into competent host cells of *E. coli* strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. The expression level of the fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The protein was in the insoluble (pellet) fraction. The cells had large inclusion bodies.

15

W. Human OPG met(22-401) (P25A)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 25 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-84 and 1289-85 were annealed to form an oligo linker duplex with XbaI and KpnI cohesive ends. The synthetic linker duplex utilized optimal *E. coli* codons and encoded an N-terminal methionine. The linker also included an SpeI restriction site which was not present in the original sequence. The linker duplex was directionally inserted between the XbaI and KpnI sites in pAMG21-huOPG-22-401 using standard methods. The ligation mixture was introduced into *E. coli* host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to

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verify the DNA sequence of the HuOPG-Met[22-401] (P25A) gene. The following oligonucleotides were used to generate the XbaI - KpnI linker:

5 Oligo #1289-84

5'-CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC
AAA ATA TCT TCA TTA TGA TGA AGA AAC TAG TCA TCA GCT GCT
GTG TGA TAA ATG TCC GCC GGG TAC -3' (SEQ ID NO:115)

10

Oligo #1289-85

5'- CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAC TAG
TTT CTT CAT CAT AAT GAA GAT ATT TTG GAG CAA AAG TTT CCA
TAT GTT ATT CCT CCT T-3' (SEQ ID NO:116)

15

X. Human OPG met[22-401] (P26A) and (P26D)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 26 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-86 and 1289-87 were annealed to form an oligo linker duplex with XbaI and SpeI cohesive ends. The synthetic linker duplex utilized optimal *E. coli* codons and encoded an N-terminal methionine. The linker duplex was directionally inserted between the XbaI and SpeI sites in pAMG21-huOPG[22-401] (P25A) using standard methods. The ligation mixture was introduced into *E. coli* host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] (P26A) gene. One of the clones sequenced was

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found to have the proline at position 26 substituted by aspartic acid rather than alanine, and this clone was designated huOPG-met[22-401] (P26D). The following oligonucleotides were used to generate the XbaI - SpeI

5 linker:

Oligo #1289-86

5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TCC
TGC TAA ATA TCT TCA TTA TGA TGA AGA AA - 3'
10 (SEQ ID NO:117)

Oligo #1289-87

5' - CTA GTT TCT TCA TCA TAA TGA AGA TAT TTA GCA
15 GGA AAA GTT TCC ATA TGT TAT TCC TCC TT - 3'
(SEQ ID NO:118)

Y Human OPG met[22-1941] (P25A)

20 A DNA sequence coding for an N-terminal methionine and amino acids 22 through 194 of human OPG with the proline at position 25 being substituted by alanine under control of the lux PR promoter in prokaryotic expression vector pAMG21 was constructed as
25 follows: The plasmids pAMG21-huOPG[27-194] and pAMG21-huOPG[22-401] (P25A) were each digested with KpnI and BamHI endonucleases. The 450 bp fragment was isolated from pAMG21-huOPG[27-194] and the 6.1 kbp fragment was isolated from pAMG21-huOPG[22-401] (P25A). These
30 fragments were ligated together and introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones

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and DNA sequencing was performed to verify the DNA sequence of the huOPG-Met[22-194] (P25A) gene.

EXAMPLE 9

5 Association of OPG Monomers

CHO cells engineered to overexpress muOPG [22-401] were used to generate conditioned media for the analysis of secreted recombinant OPG using rabbit 10 polyclonal anti-OPG antibodies. An aliquot of conditioned media was concentrated 20-fold, then analysed by reducing and non-reducing SDS-PAGE (Figure 15). Under reducing conditions, the protein migrated as a Mr 50-55 kd polypeptide, as would be 15 predicted if the mature product was glycosylated at one or more of its consensus N-linked glycosylation sites. Surprisingly, when the same samples were analysed by non-reducing SDS-PAGE, the majority of the protein migrated as an approximately 100 kd polypeptide, twice the size 20 of the reduced protein. In addition, there was a smaller amount of the Mr 50-55 kd polypeptide. This pattern of migration on SDS-PAGE was consistent with the notion that the OPG product was forming dimers through 25 oxidation of a free sulfhydryl group(s).

The predicted mature OPG polypeptide contains 23 cysteine residues, 18 of which are predicted to be involved in forming intrachain disulfide bridges which comprise the four cysteine-rich domains (Figure 12A). The five remaining C-terminal cysteine residues are not 30 involved in secondary structure which can be predicted based upon homology with other TNFR family members. Overall there is a net uneven number of cysteine residues, and it is formally possible that at least one

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residue is free to form an intermolecular disulfide bond between two OPG monomers.

To help elucidate patterns of OPG kinesis and monomer association, a pulse-chase labelling study was performed. CHO cells expressing muOPG [22-401] were metabolically labelled as described above in serum-free medium containing ^{35}S methionine and cysteine for 30 min. After this period, the media was removed, and replaced with complete medium containing unlabelled methionine and cysteine at levels approximately 2,000-fold excess to the original concentration of radioactive amino acids. At 30 min, 1hr, 2 hr, 4 hr, 6 hr and 12 hr post addition, cultures were harvested by the removal of the conditioned media, and lysates of the conditioned media and adherent monolayers were prepared. The culture media and cell lysates were clarified as described above, and then immunoprecipitated using anti-OPG antibodies as described above. After the immunoprecipitates were washed, they were released by boiling in non-reducing SDS-PAGE buffer then split into two equal halves. To one half, the reducing agent β -mercaptoethanol was added to 5% (v/v) final concentration, while the other half was maintained in non-reducing conditions. Both sets of immunoprecipitates were analysed by SDS-PAGE as described above, then processed for autoradiography and exposed to film. The results are shown in Figure 16. The samples analysed by reducing SDS-PAGE are depicted in the bottom two panels. After synthesis, the OPG polypeptide is rapidly processed to a slightly larger polypeptide, which probably represents modification by N-linked glycosylation. After approximately 1-2 hours, the level of OPG in the cell decreases dramatically, and concomitantly appears in the culture supernatant. This

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appears to be the result of the vectoral transport of OPG from the cell into the media over time, consistent with the notion that OPG is a naturally secreted protein. Analysis of the same immunoprecipitates under 5 nonreducing conditions reveals the relationship between the formation of OPG dimers and secretion into the conditioned media (Figure 16, upper panels). In the first 30-60 minutes, OPG monomers are processed in the cell by apparent glycosylation, followed by dimer 10 formation. Over time, the bulk of OPG monomers are driven into dimers, which subsequently disappear from the cell. Beginning about 60 minutes after synthesis, OPG dimers appear in the conditioned media, and accumulate over the duration of the experiment. 15 Following this period, OPG dimers are formed, which are then secreted into the culture media. OPG monomers persist at a low level inside the cell over time, and small amounts also appear in the media. This does not appear to be the result of breakdown of covalent OPG 20 dimers, but rather the production of sub-stoichiometric amounts of monomers in the cell and subsequent secretion.

Recombinantly produced OPG from transfected CHO cells appears to be predominantly a dimer. To 25 determine if dimerization is a natural process in OPG synthesis, we analysed the conditioned media of a cell line found to naturally express OPG. The CTLL-2 cell line, a murine cytotoxic T lymphocytic cell line (ATCC accession no. TIB-214), was found to express OPG mRNA in 30 a screen of tissue and cell line RNA. The OPG transcript was found to be the same as the cloned and sequenced 2.5-3.0 kb RNA identified from kidney and found to encode a secreted molecule. Western blot analysis of conditioned media obtained from CTLL-2 cells

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shows that most, if not all, of the OPG protein secreted is a dimer (Figure 17). This suggests that OPG dimerization and secretion is not an artifact of overexpression in a cell line, but is likely to be the 5 main form of the product as it is produced by expressing cells.

Normal and transgenic mouse tissues and serum were analysed to determine the nature of the OPG molecule expressed in OPG transgenic mice. Since the 10 rat OPG cDNA was expressed under the control of a hepatocyte control element, extracts made from the parenchyma of control and transgenic mice under non-reducing conditions were analysed (Figure 18). In extract from transgenic, but not control mice, OPG 15 dimers are readily detected, along with stoichiometric amounts of monomers. The OPG dimers and monomers appear identical to the recombinant murine protein expressed in the genetically engineered CHO cells. This strongly suggests that OPG dimers are 20 indeed a natural form of the gene product, and are likely to be key active components. Serum samples obtained from control and transgenic mice were similarly analysed by western blot analysis. In control mice, the majority of OPG protein migrates as a dimer, while small 25 amounts of monomer are also detected. In addition, significant amounts of a larger OPG related protein is detected, which migrates with a relative molecular mass consistent with the predicted size of a covalently-linked trimer. Thus, recombinant OPG is expressed 30 predominantly as a dimeric protein in OPG transgenic mice, and the dimer form may be the basis for the osteopetrotic phenotype in OPG mice. OPG recombinant protein may also exist in higher molecular weight "trimeric" forms.

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To determine if the five C-terminal cysteine residues of OPG play a role in homodimerization, the murine OPG codons for cysteine residues 195 (C195), 5 C202, C277, C319, and C400 were changed to serine using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as described above. The muOPG gene was subcloned between the Not I and Xba I sites of the pcDNA 3.1 (+) vector (Invitrogen, San 10 Diego, CA). The resulting plasmid, pcDNA3.1-muOPG, and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent *E. coli* XL1- 15 Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to change the codon for cysteine residue 195 to serine of the 20 murine OPG gene, resulting in the production of a muOPG [22-401] C195S protein:

1389-19:
25 5' -CAC GCA AAA GTC GGG AAT AGA TGT CAC-3'
(SEQ ID NO:150)

1406-38:
30 5' -GTG ACA TCT ATT CCC GAC TTT TGC GTG-3'
(SEQ ID NO:151)

The following primer pairs were used to change the codon for cysteine residue 202 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C202S protein:

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1389-21:

5' -CAC CCT GTC GGA AGA GGC CTT CTT C-3'
(SEQ ID NO:152)

5

1389-22:

5' -GAA GAA GGC CTC TTC CGA CAG GGT G-3' (1389-22)
(SEQ ID NO:153)

10

The following primer pairs were used to change the codon for cysteine residue 277 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C277S protein:

15 1389-23:

5' -TGA CCT CTC GGA AAG CAG CGT GCA-3'
(SEQ ID NO:154)

1389-24:

20 5' -TGC ACG CTG CTT TCC GAG AGG TCA-3'
(SEQ ID NO:155)

25

The following primer pairs were used to change the codon for cysteine residue 319 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C319S protein:

1389-17:

30 5' -CCT CGA AAT CGA GCG AGC AGC TCC-3'
(SEQ ID NO:156)

1389-18:

5' -CGA TTT CGA GGT CTT TCT CGT TCT C-3'
(SEQ ID NO:157)

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The following primer pairs were used to change
the codon for cysteine residue 400 to serine of the
murine OPG gene, resulting in the production of a muOPG
5 [22-401] C400S protein:

1406-72:

5' -CCG TGA AAA TAA GCT CGT TAT AAC TAG GAA TGG-3'
(SEQ ID NO:158)

10

1406-75:

5' -CCA TTC CTA GTT ATA ACG AGC TTA TTT TCA CGG-3'
(SEQ ID NO:159)

15

Each resulting muOPG [22-401] plasmid
containing the appropriate mutation was then transfected
into human 293 cells, the mutant OPG-Fc fusion protein
purified from conditioned media as described above. The
biological activity of each protein was assessed the in
vitro osteoclast forming assay described in example 11.
Conditioned media from each transfectant was analysed by
non-reducing SDS-PAGE and western blotting with anti-OPG
20 25 antibodies.

Mutation of any of the five C-terminal
cysteine residues results in the production of
predominantly (>90%) monomeric 55 kd OPG molecules.
This strongly suggests that the C-terminal cysteine
residues together play a role in OPG homodimerization.

30 C-terminal OPG deletion mutants were
constructed to map the region(s) of the OPG C-terminal
domain which are important for OPG homodimerization.
These OPG mutants were constructed by PCR amplification
using primers which introduce premature stop translation

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signals in the C-terminal region of murine OPG. The 5' oligo was designed to the MuOPG start codon (containing a HindIII restriction site) and the 3' oligonucleotides (containing a stop codon and XbaI site) were designed to 5 truncate the C-terminal region of muOPG ending at either threonine residue 200 (CT 200), proline 212 (CT212), glutamic acid 293 (CT-293), or serine 355 (CT-355).
The following primers were used to construct muOPG [22-200]:

10

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:160)

15

1391-91:

5' -CCT CTC TCG AGT CAG GTG ACA TCT ATT CCA CAC TTT
TGC GTG GC-3' (1391-91) (SEQ ID NO:161)

20

The following primers were used to construct muOPG [22-212]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
25 AG-3' (SEQ ID NO:162)

1391-90:

5' -CCT CTC TCG AGT CAA GGA ACA GCA AAC CTG AAG AAG
GC -3' (SEQ ID NO:163)

30

The following primers were used to construct muOPG [22-293]:

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1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:164)

1391-89:

5

5'- CCT CTC TCG AGT CAC TCT GTG GTG AGG TTC GAG TGG
CC-3' (SEQ ID NO:165)

10 The following primers were used to construct muOPG
[22-355]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:166)

15

1391-88:

5' CCT CTC TCG AGT CAG GAT GTT TTC AAG TGC TTG AGG GC-3'
(SEQ ID NO:167)

20

Each resulting muOPG-CT plasmid containing the appropriate truncation was then transfected into human 293 cells, the mutant OPG-FC fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11. The conditioned medias were also analysed by non-reducing SDS-PAGE and western blotting using anti-OPG antibodies.

30

Truncation of the C-terminal region of OPG effects the ability of OPG to form homodimers. CT 355 is predominantly monomeric, although some dimer is formed. CT 293 forms what appears to be equal molar amounts of monomer and dimer, and also high molecular

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weight aggregates. However, CT 212 and CT 200 are monomeric.

EXAMPLE 10

5

Purification of OPG

A. Purification of mammalian OPG-Fc Fusion Proteins

5 L of conditioned media from 293 cells expressing an OPG-Fc fusion protein were prepared as follows. A frozen sample of cells was thawed into 10 ml of 293S media (DMEM-high glucose, 1x L-glutamine, 10% heat inactivated fetal bovine serum (FBS) and 100 ug/ml hygromycin) and fed with fresh media after one day. After three days, cells were split into two T175 flasks at 1:10 and 1:20 dilutions. Two additional 1:10 splits were done to scale up to 200 T175 flasks. Cells were at 5 days post-thawing at this point. Cells were grown to near confluence (about three days) at which time serum-containing media was aspirated, cells were washed one time with 25 ml PBS per flask and 25 ml of SF media (DMEM-high glucose, 1x L-glutamine) was added to each flask. Cells were maintained at 5% CO₂ for three days at which point the media was harvested, centrifuged, and filtered through 0.45m cellulose nitrate filters (Corning).

OPG-Fc fusion proteins were purified using a Protein G Sepharose column (Pharmacia) equilibrated in PBS. The column size varied depending on volume of starting media. Conditioned media prepared as described above was loaded onto the column, the column washed with PBS, and pure protein eluted using 100mM glycine pH 2.7. Fractions were collected into tubes containing 1M Tris pH 9.2 in order to neutralize as quickly as possible. Protein containing fractions were pooled, concentrated

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in either an Amicon Centricon 10 or Centriprep 10 and diafiltered into PBS. The pure protein is stored at - 80°C.

5 Murine [22-401]-Fc, Murine [22-180]-Fc, Murine (22-194)-Fc, human [22-401]-Fc and human [22-201]Fc were purified by this procedure. Murine [22-185]-Fc is purified by this procedure.

B. Preparation of anti-OPG antibodies

10 Three New Zealand White rabbits (5-8 lbs initial wt) were injected subcutaneously with muOPG(22-401)-Fc fusion protein. Each rabbit was immunized on day 1 with 50 µg of antigen emulsified in an equal volume of Freunds complete adjuvant. Further boosts 15 (Days 14 and 28) were performed by the same procedure with the substitution of Freunds incomplete adjuvant. Antibody titers were monitored by EIA. After the second boost, the antisera revealed high antibody titers and 25ml production bleeds were obtained from each animal.

20 The sera was first passed over an affinity column to which murine OPG-Fc had be immobilized. The anti-OPG antibodies were eluted with Pierce Gentle Elution Buffer containing 1% glacial acetic acid. The eluted protein was then dialyzed into PBS and passed over a Fc column 25 to remove any antibodies specific for the Fc portion of the OPG fusion protein. The run through fractions containing anti-OPG specific antibodies were dialyzed into PBS.

30 C. Purification of murine OPG[22-401]

Antibody Affinity Chromatography

Affinity purified anti-OPG antibodies were diafiltrated into coupling buffer (0.1M sodium carbonate

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pH 8.3, 0.5M NaCl), and mixed with CNBr-activated sepharose beads (Pharmacia) for two hours at room temperature. The resin was then washed with coupling buffer extensively before blocking unoccupied sited with 5 1M ethanolamine (pH 8.0) for two hours at room temperature. The resin was then washed with low pH (0.1M sodium acetate pH 4.0, 0.5M NaCl) followed by a high pH wash (0.1M Tris-HCl pH 8.0, 0.5M NaCl). The last washes were repeated three times. The resin was finally 10 equilibrated with PBS before packing into a column. Once packed, the resin was washed with PBS. A blank elution was performed with 0.1M glycine-HCl, pH 2.5), followed by re-equilibration with PBS.

Concentrated conditioned media from CHO cells expressing muOPG[22-410] was applied to the column at a 15 low flow rate. The column was washed with PBS until UV absorbance measured at 280nm returned to baseline. The protein was eluted from the column first with 0.1M glycine-HCl (pH 2.5), re-equilibrated with PBS, and 20 eluted with a second buffer (0.1M CAPS, pH 10.5), 1M NaCl). The two elution pools were diafiltered separately into PBS and sterile filtered before freezing at -20°C.

Conventional Chromatography

CHO cell conditioned media was concentrated 25 23x in an Amicon spiral wound cartridge (S10Y10) and diafiltrated into 20mM tris pH 8.0. The diafiltrated media was then applied to a Q-sepharose HP (Pharmacia) column which had been equilibrated with 20mM tris pH 8.0. The 30 column was then washed until absorbence at 280nm reached baseline. Protein was eluted with a 20 column volume gradient of 0-300mM NaCl in tris pH 8.0. OPG protein was detected using a western blot of column fractions.

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Fractions containing OPG were pooled and brought to a final concentration of 300mM NaCl, 0.2mM DTT. A NiNTA superose (Qiagen) column was equilibrated with 20mM tris pH 8.0, 300mM NaCl, 0.2mM DTT after which

- 5 the pooled fractions were applied. The column was washed with equilibration buffer until baseline absorbence was reached. Proteins were eluted from the column with a 0-30mM Imidazole gradient in equilibration buffer. Remaining proteins were washed off the column with 1M Imidazole. Again a western blot was used to detect OPG containing fractions.

Pooled fractions from the NiNTA column were dialyzed into 10mm potassium phosphate pH 7.0, 0.2mM DTT. The dialyzed pool was then applied to a ceramic 15 hydroxyapatite column (Bio-Rad) which had been equilibrated in 10mM phosphate buffer. After column washing, the protein was eluted with a 10-100mM potassium phosphate gradient over 20 column volumes. This was then followed by a 20 column volume gradient of 20 100-400 mM phosphate.

OPG was detected by coomassie blue staining of SDS-polyacrylamide gels and by western blotting. Fractions were pooled and diafiltered onto PBS and frozen at -80°C. The purified protein runs as a monomer 25 and will remain so after diafiltration into PBS. The monomer is stable when stored frozen or at pH 5 at 4°C. However if stored at 4°C in PBS, dimers and what appears to be trimers and tetramers will form after one week.

30

D. Purification of human OPG met[22-401] from E. coli

The bacterial cell paste was suspended into 10 mM EDTA to a concentration of 15% (w/v) using a low shear homogenizer at 5°C. The cells were then disrupted

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by two homogenizations at 15,000 psi each at 5°C. The resulting homogenate was centrifuged at 5,000 x g for one hour at 5°C. The centrifugal pellet was washed by low shear homogenization into water at the original 5 homogenization volume followed by centrifugation as before. The washed pellet was then solubilized to 15% (w/v) by a solution of (final concentration) 6 M guanidine HCl, 10 mM dithiothreitol, 10 mM TrisHCl, pH 8.5 at ambient temperature for 30 minutes. This 10 solution was diluted 30-fold into 2M urea containing 50 mM CAPS, pH 10.5, 1 mM reduced glutathione and then stirred for 72 hours at 5°C. The OPG was purified from this solution at 25°C by first adjustment to pH 4.5 with acetic acid and then chromatography over a column of 15 SP-HP Sepharose resin equilibrated with 25 mM sodium acetate, pH 4.5. The column elution was carried out with a linear sodium chloride gradient from 50 mM to 550 mM in the same buffer using 20 column volumes at a flow rate of 0.1 column volumes/minute. The peak fractions 20 containing only the desired OPG form were pooled and stored at 5°C or buffer exchanged into phosphate buffered saline, concentrated by ultrafiltration, and then stored at 5°C. This material was analyzed by reverse phase HPLC, SDS-PAGE, limulus amebocyte lysate 25 assay for the presence of endotoxin, and N-terminal sequencing. In addition, techniques such as mass spectrometry, pH/temperature stability, fluorescence, circular dichroism, differential scanning calorimetry, and protease profiling assays may also be used to 30 examine the folded nature of the protein.

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EXAMPLE 11
Biological Activity of Recombinant OPG

Based on histology and histomorphometry, it
5 appeared that hepatic overexpression of OPG in
transgenic mice markedly decreased the numbers of
osteoclasts leading to a marked increase in bone tissue
(see Example 4). To gain further insight into potential
mechanism(s) underlying this *in vivo* effect, various
10 forms of recombinant OPG have been tested in an *in vitro*
culture model of osteoclast formation (osteoclast
forming assay). This culture system was originally
devised by Udagawa (Udagawa et al. Endocrinology 125,
1805-1813 (1989), Proc. Natl. Acad. Sci. USA 87, 7260-
15 7264 (1990)) and employs a combination of bone marrow
cells and cells from bone marrow stromal cell lines. A
description of the modification of this culture system
used for these studies has been previously published
(Lacey et al. Endocrinology 136, 2367-2376 (1995)). In
20 this method, bone marrow cells, flushed from the femurs
and tibiae of mice, are cultured overnight in culture
media (alpha MEM with 10% heat inactivated fetal bovine
serum) supplemented with 500 U/ml CSF-1 (colony
stimulating factor 1, also called M-CSF), a
25 hematopoietic growth factor specific for cells of the
monocyte/macrophage family lineage. Following this
incubation, the non-adherent cells are collected,
subjected to gradient purification, and then cocultured
with cells from the bone marrow cell line ST2 (1×10^6
30 non-adherent cells : 1×10^5 ST2 cells/ ml media). The
media is supplemented with dexamethasone (100 nM) and
the biologically-active metabolite of vitamin D3 known
as 1,25 dihydroxyvitamin D3 (1,25 (OH)₂ D₃, 10 nM). To
enhance osteoclast appearance, prostaglandin E2 (250 nM)

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is added to some cultures. The coculture period usually ranges from 8 - 10 days and the media, with all of the supplements freshly added, is renewed every 3-4 days.

5 At various intervals, the cultures are assessed for the presence of tartrate acid phosphatase (TRAP) using either a histochemical stain (Sigma Kit # 387A, Sigma, St. Louis, MO) or TRAP solution assay. The TRAP histochemical method allows for the identification of osteoclasts phenotypically which are multinucleated (≥ 3 nuclei) cells that are also TRAP+. The solution assay involves lysing the osteoclast-containing cultures in a citrate buffer (100 mM, pH 5.0) containing 0.1% Triton X-100. Tartrate resistant acid phosphatase activity is then measured based on the conversion of

10 p-nitrophenylphosphate (20 nM) to p-nitrophenol in the presence of 80 mM sodium tartrate which occurs during a 3-5 minute incubation at RT. The reaction is terminated by the addition of NaOH to a final concentration of 0.5 M. The optical density at 405 nm is measured and the

15 results are plotted.

20

Previous studies (Udagawa et al. *ibid*) using the osteoclast forming assay have demonstrated that these cells express receptors for ^{125}I -calcitonin (autoradiography) and can make pits on bone surfaces,

25 which when combined with TRAP positivity confirm that the multinucleated cells have an osteoclast phenotype. Additional evidence in support of the osteoclast phenotype of the multinucleated cells that arise *in vitro* in the osteoclast forming assay are that the cells express αv and $\beta 3$ integrins by immunocytochemistry and calcitonin receptor and TRAP mRNA by *in situ* hybridization (ISH).

30

The huOPG [22-401]-Fc fusion was purified from CHO cell conditioned media and subsequently utilized in

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- the osteoclast forming assay. At 100 ng/ml of huOPG [22-401]-Fc, osteoclast formation was virtually 100% inhibited (Figure 19A). The levels of TRAP measured in lysed cultures in microtitre plate wells were also inhibited in the presence of OPG with an ID₅₀ of approximately 3 ng/ml (Figure 20). The level of TRAP activity in lysates appeared to correlate with the relative number of osteoclasts seen by TRAP cytochemistry (compare Figures 19A-19G and 20).
- 5 Purified human IgG1 and TNFbp were also tested in this model and were found to have no inhibitory or stimulatory effects suggesting that the inhibitory effects of the huOPG [22-401]-Fc were due to the OPG portion of the fusion protein.
- 10 Additional forms of the human and murine molecules have been tested and the cumulative data are summarized in Table 1.
- 15

Table 1
Effects of various OPG forms on in vitro
osteoclast formation

	<u>OPG Construct</u>	<u>Relative Bioactivity in vitro</u>
20		
	muOPG [22-401]-Fc	+++
	muOPG [22-194]-Fc	+++
	muOPG [22-185]-Fc	++
	muOPG [22-180]-Fc	-
25	muOPG [22-401]	+++
	muOPG [22-401] C195	+++
	muOPG [22-401] C202	+
	muOPG [22-401] C277	-
30	muOPG [22-401] C319	+

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	muOPG [22-401] C400	+
	muOPG [22-185]	-
	muOPG [22-194]	++
	muOPG [22-200]	++
5	muOPG [22-212]	-
	muOPG [22-293]	+++
	muOPG [22-355]	+++
	huOPG [22-401]-Fc	+++
10	huOPG [22-201]-Fc	+++
	huOPG [22-401]-Fc P26A	+++
	huOPG [22-401]-Fc Y28F	+++
	huOPG [22-401]	+++
	huOPG [27-401]-Fc	++
15	huOPG [29-401]-Fc	++
	huOPG [32-401]-Fc	+/-
	+++ , ED ₅₀ = 0.4-2 ng/ml	
	++, ED ₅₀ = 2-10 ng/ml	
20	+, ED ₅₀ = 10-100 ng/ml	
	- , ED ₅₀ > 100 ng/ml	

The cumulative data suggest that murine and human OPG amino acid sequences 22-401 are fully active *in vitro*, when either fused to the Fc domain, or unfused. They inhibit in a dose-dependent manner and possess half-maximal activities in the 2-10 ng/ml range. Truncation of the murine C-terminus at threonine residue 180 inactivates the molecule, whereas truncations at cysteine 185 and beyond have full activity. The cysteine residue located at position 185 is predicted to form an SS3 bond in the domain 4 region of OPG. Removal of this residue in other TNFR-related proteins has previously been shown to abrogate biological activity.

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(Yan et al. J. Biol. Chem. 266, 12099-12104 (1994)). Our finding that muOPG[22-180]-Fc is inactive while muOPG[22-185]-Fc is active is consistent with these findings. This suggests that amino acid residues 22-185 define a region for OPG activity.

These findings indicate that like transgenically-expressed OPG, recombinant OPG protein also suppressed osteoclast formation as tested in the osteoclast forming assay. Time course experiments examining the appearance of TRAP+ cells, β 3+ cells, F480+ cells in cultures continuously exposed to OPG demonstrate that OPG blocks the appearance TRAP+ and β 3+ cells, but not F480+ cells. In contrast, TRAP+ and β 3+ cells begin to appear as early as day 4 following culture establishment in control cultures. Only F480+ cells can be found in OPG-treated cultures and they appear to be present at qualitatively the same numbers as the control cultures. Thus, the mechanism of OPG effects in vitro appears to involve a blockade in osteoclast differentiation at a step beyond the appearance of monocyte-macrophages but before the appearance of cells expressing either TRAP or β 3 integrins. Collectively these findings indicate that OPG does not interfere with the general growth and differentiation of monocyte-macrophage precursors from bone marrow, but rather suggests that OPG specifically blocks the selective differentiation of osteoclasts from monocyte-macrophage precursors.

To determine more specifically when in the osteoclast differentiation pathway that OPG was inhibitory, a variation of the in vitro culture method was employed. This variation, described in (Lacey et al. *supra*), employs bone marrow macrophages as osteoclast precursors. The osteoclast precursors are

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derived by taking the nonadherent bone marrow cells after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000 - 2,000 U/ml CSF-1. Following 4 days of culture, termed 5 the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/ml CSF-1. This 2 day period is called the intermediate 10 differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells (1×10^5 cell/ml), dexamethasone (100 nM) and 1,25 (OH)₂ D₃ (10 nM) are added for the last 8 days for what is termed the terminal differentiation period. Test agents can be 15 added during this terminal period as well. Acquisition of phenotypic markers of osteoclast differentiation are acquired during this terminal period (Lacey et al. *ibid*).

20 huOPG [22-401]-Fc (100 ng/ml) was tested for its effects on osteoclast formation in this model by adding it during either the intermediate, terminal or, alternatively, both differentiation periods. Both TRAP cytochemistry and solution assays were performed. The results of the solution assay are shown in Figure 21. 25 HuOPG [22-401]-Fc inhibited the appearance of TRAP activity when added to both the intermediate and terminal or only the terminal differentiation phases. When added to the intermediate phase and then removed from the cultures by rinsing, huOPG [22-401]-Fc did not 30 block the appearance of TRAP activity in culture lysates. The cytochemistry results parallel the solution assay data. Collectively, these observations indicate that huOPG [22-401]-Fc only needs to be present during the terminal differentiation period for it to

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exert its all of its suppressive effects on osteoclast formation.

B. In vivo IL1- α and IL1- β challenge experiments

IL1 increases bone resorption both
5 systemically and locally when injected subcutaneously over the calvaria of mice (Boyce et al., Endocrinology 125, 1142-1150 (1989)). The systemic effects can be assessed by the degree of hypercalcemia and the local effects histologically by assessing the relative
10 magnitude of the osteoclast-mediated response. The aim of these experiments was to determine if recombinant muOPG [22-401]-Fc could modify the local and/or systemic actions of IL1 when injected subcutaneously over the same region of the calvaria as IL1.
15

IL-1 β experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group: IL1 treated animals (mice received 1 injection/day of 2.5 μ g of IL1- β); Low dose muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 1 μ g of muOPG [22-401]-Fc); Low dose muOPG [22-401]-Fc and IL1- β ; High dose muOPG [22-401]-Fc treated animals (mice receive 3 injections/day of 10 μ g muOPG [22-401]-Fc); High dose muOPG [22-401]-Fc and IL1- β . All mice received the same total number of injections of either active factor or vehicle (0.1% bovine serum albumin in phosphate buffered saline). All groups are sacrificed on the day after the last
25 injection. The weights and blood ionized calcium levels are measured before the first injections, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed.
30

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After sacrifice the calvaria were removed and processed for paraffin sectioning.

III- α experiment

5 Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group; III alpha treated animals (mice received 1 injection/day of 5 μ g of III-alpha); Low dose muOPG [22-401]-Fc treated animals (mice received 1
10 injection/day of 10 μ g of muOPG [22-401]-Fc; Low dose muOPG [22-401]-Fc and III-alpha, (dosing as above); High dose muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 10 μ g muOPG [22-401]-Fc; High dose muOPG [22-401]-Fc and III- α . All mice received the same
15 number of injections/day of either active factor or vehicle. All groups were sacrificed on the day after the last injection. The blood ionized calcium levels were measured before the first injection, four hours after the second injection and 24 hours after the third
20 III injection, just before the animals were sacrificed. The animal weights were measured before the first injection, four hours after the second injection and 24 hours after the third III injection, just before the animals were sacrificed. After sacrifice the calvaria
25 were removed and processed for paraffin sectioning.

Histological methods

30 Calvarial bone samples were fixed in zinc formalin, decalcified in formic acid, dehydrated through ethanol and mounted in paraffin. Sections (5 μ m thick) were cut through the calvaria adjacent to the lambdoid suture and stained with either hematoxylin and eosin or reacted for tartrate resistant acid phosphatase activity

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(Sigma Kit# 387A) and counterstained with hematoxylin. Bone resorption was assessed in the IL1- α treated mice by histomorphometric methods using the Osteomeasure (Osteometrics, Atlanta, GA) by tracing histologic features onto a digitizer platen using a microscope-mounted camera lucida attachment. Osteoclast numbers, osteoclast lined surfaces, and eroded surfaces were determined in the marrow spaces of the calvarial bone. The injected and non-injected sides of the calvaria were measured separately.

Results

IL1- α and IL1- β produced hypercalcemia at the doses used, particularly on the second day, presumably by the induction of increased bone resorption systemically. The hypercalcemic response was blocked by muOPG [22-401]-Fc in the IL1-beta treated mice and significantly diminished in mice treated with IL1-alpha, an effect most apparent on day 2 (Figure 22A-22B).

Histologic analysis of the calvariae of mice treated with IL1-alpha and beta shows that IL1 treatments alone produce a marked increase in the indices of bone resorption including: osteoclast number, osteoclast lined surface, and eroded surface (surfaces showing deep scalloping due to osteoclastic action (Figure 23B, Table 2). In response to IL1- α or IL1- β , the increases in bone resorption were similar on the injected and non-injected sides of the calvaria. Muopg [22-401]-Fc injections reduced bone resorption in both IL1-alpha and beta treated mice and in mice receiving vehicle alone but this reduction was seen only on the muopg [22-401]-Fc injected sides of the calvariae.

The most likely explanation for these observations is that muOPG [22-401]-Fc inhibited bone

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resorption, a conclusion supported by the reduction of both the total osteoclast number and the percentage of available bone surface undergoing bone resorption, in the region of the calvaria adjacent to the muOPG [22-401]-Fc injection sites. The actions of muOPG [22-401]-Fc appeared to be most marked locally by histology, but the fact that muOPG [22-401]-Fc also blunted IL1-induced hypercalcemia suggests that muOPG [22-401]-Fc has more subtle effects on bone resorption systemically.

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Table 2: Effects of OPG on variables of bone resorption in IL-1 injected mice.

	Osteoclast Surface & Bone Surface (mean ± S.D.)		Eroded Surface & Bone Surface (mean ± S.D.)		Osteoclast Number/mm ² Tissue Area (mean ± S.D.)	
	Non-injected side	Injected side	Non-injected side	Injected side	Non-injected side	Injected side
Experiment 1						
Control	12.36 ± 3.44	9.54 ± 2.46	8.07 ± 3.90	9.75 ± 3.16	32.51 ± 11.09	23.50 ± 10.83
IL-1-β (2.5μg/d)	17.18 ± 1.30	16.40 ± 2.16	40.66 ± 4.28	37.53 ± 10.28	71.80 ± 18.76	60.89 ± 5.16
OPG (40μg/d)	10.12 ± 3.71	5.04 ± 1.66	9.73 ± 4.33	4.19 ± 3.61	32.73 ± 11.09	15.24 ± 7.54
OPG+IL-1-β	18.61 ± 2.46	6.13.26 ± 2.50	44.87 ± 8.63	# 25.94 ± 6.82	69.42 ± 36.29	# 47.13 ± 24.26
Experiment 2						
Control	11.56 ± 4.22	11.95 ± 2.97	12.67 ± 5.04	10.03 ± 5.13	51.72 ± 23.93	56.03 ± 30.70
IL-1-α (5μg/d)	28.81 ± 4.84	23.46 ± 5.76	37.51 ± 5.16	41.10 ± 12.53	113.60 ± 18.04	102.70 ± 32.09
OPG (40μg/d)	14.40 ± 1.00	# 4.26 ± 2.54	11.55 ± 4.14	# 4.29 ± 3.16	72.28 ± 14.11	# 22.65 ± 16.68
OPG+IL-1-α	29.58 ± 8.80	# 17.83 ± 3.34	33.66 ± 9.21	# 24.38 ± 8.88	146.10 ± 42.37	# 66.56 ± 15.62

Different to non-injected side p < 0.05 (by paired t test)

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C. Systemic Effects of muOPG [22-401]-Fc in Growing Mice

Male BDF1 mice aged 3-4 weeks, weight range 9.2- 15.7g were divided into groups of ten mice per group. These mice were injected subcutaneously with 5 saline or muOPG [22-401]-Fc 2.5mg/kg bid for 14 days (5mg/kg/day). The mice were radiographed before treatment, at day 7 and on day 14. The mice were sacrificed 24 hours after the final injection. The right femur was removed, fixed in zinc formalin, 10 decalcified in formic acid and embedded in paraffin. Sections were cut through the mid region of the distal femoral metaphysis and the femoral shaft. Bone density, by histomorphometry, was determined in six adjacent regions extending from the metaphyseal limit of the 15 growth plate, through the primary and secondary spongiosa and into the femoral diaphysis (shaft). Each region was 0.5 x 0.5 mm².

Radiographic changes

20 After seven days of treatment there was evidence of a zone of increased bone density in the spongiosa associated with the growth plates in the OPG treated mice relative to that seen in the controls. The effects were particularly striking in the distal femoral 25 and the proximal tibial metaphyses (Figure 24A-24B). However bands of increased density were also apparent in the vertebral bodies, the iliac crest and the distal tibia. At 14 days, the regions of opacity had extended further into the femoral and tibial shafts though the 30 intensity of the radio-opacity was diminished. Additionally, there were no differences in the length of the femurs at the completion of the experiment or in the change in length over the duration of the experiment implying that OPG does not alter bone growth.

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Histological Changes

The distal femoral metaphysis showed increased bone density in a regions 1.1 to 2.65 mm in distance from the growth plate (Figures 25 and 26A-26B). This is a region where bone is rapidly removed by osteoclast-mediated bone resorption in mice. In these rapidly growing young mice, the increase in bone in this region observed with OPG treatment is consistent with an inhibition of bone resorption.

D. Effects of Osteoprotegerin on Bone Loss Induced by Ovariectomy in the Rat

Twelve week old female Fisher rats were ovariectomized (OVX) or sham operated and dual xray absorptiometry (DEXA) measurements made of the bone density in the distal femoral metaphysis. After 3 days recovery period, the animals received daily injections for 14 days as follows: Ten sham operated animals received vehicle (phosphate buffered saline); Ten OVX animals received vehicle (phosphate buffered saline); Six OVX animals received OPG-Fc 5mg/kg SC; Six OVX animals received pamidronate (PAM) 5mg/kg SC; Six OVX animals received estrogen (ESTR) 40ug/kg SC. After 7 and 14 days treatment the animals had bone density measured by DEXA. Two days after the last injection the animals were killed and the right tibia and femur removed for histological evaluation.

The DEXA measurements of bone density showed a trend to reduction in the bone density following ovariectomy that was blocked by OPG-Fc. Its effects were similar to the known antiresorptive agents estrogen and pamidronate. (Figure 27). The histomorphometric

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analysis confirmed these observations with OPG-Fc treatment producing a bone density that was significantly higher in OVX rats than that seen in untreated OVX rats (Figure 28). These results confirm 5 the activity of OPG in the bone loss associated with withdrawal of endogenous estrogen following ovariectomy.

In vivo Summary

10 The in vivo actions of recombinant OPG parallel the changes seen in OPG transgenic mice. The reduction in osteoclast number seen in the OPG transgenic is reproduced by injecting recombinant OPG locally over the calvaria in both normal mice and in 15 mice treated with IL1- α or IL1- β . The OPG transgenic mice develop an osteopetrotic phenotype with progressive filling of the marrow cavity with bone and unremodelled cartilage extending from the growth plates from day 1 onward after birth. In normal three week old (growing) 20 mice, OPG treatments also led to retention of bone and unremodelled cartilage in regions of endochondral bone formation, an effect observed radiographically and confirmed histologically. Thus, recombinant OPG produces phenotypic changes in normal animals similar to 25 those seen in the transgenic animals and the changes are consistent with OPG-induced inhibition of bone resorption. Based on *in vitro* assays of osteoclast formation, a significant portion of this inhibition is due to impaired osteoclast formation. Consistent with this hypothesis, OPG blocks ovariectomy-induced 30 osteoporosis in rat. Bone loss in this model is known to be mediated by activated osteoclasts, suggesting a role for OPG in treatment of primary osteoporosis.

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EXAMPLE 12
Pegylation Derivatives of OPG

5 Preparation of N-terminal PEG-OPG conjugates by reductive alkylation

HuOPG met [22-194] P25A was buffer exchanged into 25-50 mM NaOAc, pH 4.5-4.8 and concentrated to 2-5 mg/ml. This solution was used to conduct OPG reductive alkylation with monofunctional PEG aldehydes at 5-7 °C. 10 PEG monofunctional aldehydes, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 2-4 moles of PEG aldehyde per mole of OPG. 15 After dissolution of polymer into the protein solution, sodium cyanoborohydride was added to give a final concentration of 15 to 20 mM in the reaction mixture from 1-1.6 M freshly prepared stock solution in cold DI water. The progress of the reaction and the extent of 20 OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO₄, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 16-18 hours, after which the reaction mixture was diluted 6-8 times and the pH 25 lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- 30 PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE. By N-terminal sequencing, it was determined that the monoPEG-OPG conjugate, the major reaction product in most cases, was 98% N-terminally PEG-modified OPG.

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This procedure was generally used to prepare the following N-terminal PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A: 5 kD monoPEG, 10 kD mono branched PEG, 12 kD monoPEG, 20 kD monoPEG, 20 kD mono branched PEG, 25 kD monoPEG, 31 kD monoPEG, 57 kD monoPEG, 12 kD diPEG, 25 kD diPEG, 31 kD diPEG, 57 kD diPEG, 25 kD triPEG.

Preparation of PEG-OPG conjugates by acylation

HuOPG met [22-194] P25A was buffer exchanged into 50 mM BICINE buffer, pH 8 and concentrated to 2-3 mg/ml. This solution was used to conduct OPG acylation with monofunctional PEG N-hydroxysuccinimidyl esters at room temperature. PEG N-hydroxysuccinimidyl esters, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 4-8 moles of PEG N-hydroxysuccinimidyl ester per mole of OPG. The progress of the reaction and the extent of OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO₄, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 1 hour, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE.

This procedure was generally used to prepare the following PEG-OPG conjugates: 5 kD polyPEG, 20 kD polyPEG, 40 kD poly branched PEG, 50 kD poly PEG.

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Preparation of dimeric PEG-OPG

- HuOPG met [22-194] P25A is prepared for
5 thiolation at 1-3 mg/ml in a phosphate buffer at near
neutral pH. S-acetyl mecaptosuccinic anhydride (AMSA) is
added in a 3-7 fold molar excess while maintaining pH at
7.0 and the rxn stirred at 4°C for 2 hrs. The
monothiolated-OPG is separated from unmodified and
10 polythiolated OPG by ion exchange chromatography and the
protected thiol deprotected by treatment with
hydroxylamine. After deprotection, the hydroxylamine is
removed by gel filtration and the resultant
monothiolated-OPG is subjected to a variety of thiol
15 specific crosslinking chemistries. To generate a
disulfide bonded dimer, the thiolated OPG at >1mg/ml is
allowed to undergo air oxidation by dialysis in slightly
basic phosphate buffer. The covalent thioether OPG
dimer was prepared by reacting the bis-maleimide
20 crosslinker, N,N-bis(3-maleimido propianyl)-2-hydroxy
1,3 propane with the thiolated OPG at >1mg/ml at a 0.6x
molar ratio of crosslinker:OPG in phosphate buffer at pH
6.5. Similarly, the PEG dumbbells are produced by
reaction of stoichiometric amounts of bis-maleimide
25 PEG crosslinkers with thiolated OPG at >1mg/ml in
phosphate buffer at pH 6.5. Any of the above dimeric
conjugates may be further purified using either ion
exchange or size exclusion chromatographies.
Dimeric PEG-OPG conjugates (where OPG is HuOPG met
30 [22-194] P25A prepared using the above procedures
include disulfide-bonded OPG dimer, covalent thioether
OPG dimer with an aliphatic amine type crosslinker, 3.4
kD and 8kD PEG dumbbells and monobells.

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PEG-OPG conjugates were tested for activity in vitro using the osteoclast maturation assay described in Example 11A and for activity in vivo by measuring increased bone density after injection into mice as 5 described in Example 11C. The in vivo activity is shown below in Table 3.

Table 3
In vivo biological activity of Pegylated OPG

	<u>OPG Construct</u>	<u>Increase in Tibial Bone Density</u>
	muOPG met [22-194]	-
10	muOPG met [22-194] 5k PEG	+
	muOPG met [22-194] 20k PEG	+
	huOPG met [22-194] P25A	-
15	huOPG met [22-194] P25A 5k PEG	+
	huOPG met [22-194] P25A 20k PEG	+
20	huOPG met [22-194] P25A 31k PEG	+
	huOPG met [22-194] P25A 57k PEG	+
	huOPG met [22-194] P25A 12k PEG	+
	huOPG met [22-194] P25A 20k Branched PEG	+
25	huOPG met [22-194] P25A 8k PEG dimer	+
	huOPG met [22-194] P25A disulfide crosslink	+

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* * *

While the invention has been described in what
is considered to be its preferred embodiments, it is not
5 to be limited to the disclosed embodiments, but on the
contrary, is intended to cover various modifications and
equivalents included within the spirit and scope of the
 appended claims, which scope is to be accorded the
 broadest interpretation so as to encompass all such
10 modifications and equivalents.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Amgen Inc.

10

(ii) TITLE OF INVENTION: OSTEOPROTEGERIN

(iii) NUMBER OF SEQUENCES: 168

15

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Amgen Inc.
- (B) STREET: 1840 Dehavenland Drive
- (C) CITY: Thousand Oaks
- (D) STATE: California
- (E) COUNTRY: United States
- (F) ZIP: 91320

20

(v) COMPUTER READABLE FORM:

- 25
- (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Winter, Robert B.
- (C) REFERENCE/DOCKET NUMBER: A-378-CIP2

40

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

45

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5 AAAGGAAGGA AAAAAGCGGC CGCTACANNN NNNNNNT

36

(2) INFORMATION FOR SEQ ID NO:2:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCCACG CGTCCG

16

25 (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTGCGCAG GC

40

12

(2) INFORMATION FOR SEQ ID NO:4:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

5 TGTAAAACGA CGGCCAGT

18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGGAAACAG CTATGACC

18

(2) INFORMATION FOR SEQ ID NO:6:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAATTAAACCC TCACTAAAGG

20

40 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

- 148 -

GCATTATGAC CCAGAAACCG GAC

5 (2) INFORMATION FOR SEQ ID NO:8:

23

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
10 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGTAGCGCC CTTCCCTACA TTC

20 (2) INFORMATION FOR SEQ ID NO:9:

23

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACTAGTCCC ACAATGAACA AGTGGCTGTG

30

35 (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
40 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAAGAACATGC GGCGCGTAAA CTATGAAACA GCCCAGTGAC CATTC

45

50 (2) INFORMATION FOR SEQ ID NO:11:

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- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCTCTAGAA AGAGCTGGGA C

21

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:

 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO.12.

CGCCCGTGTTC CATTATGAG C

21

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:

 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13.

ATCAAAGGCA GGGCTTCTGCCTGCGA

24

45 (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTGCACTCC TGTTTCACGG TCTG

10 10 (2) INFORMATION FOR SEQ ID NO:15:

24

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAAGACACCT TGAAGGGCCT GATG

25

24

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAACTTTTAC AGAAGAGCAT CAGC

40

24

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

- 151 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCGCGGCCG CATGAACAAG TGGCTGTGCT GCG

33

(2) INFORMATION FOR SEQ ID NO:18:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTCTAGAG AACACAGCCCA GTGACCATTG C

31

25 (2) INFORMATION FOR SEQ ID NO:19:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

40 GTGAAGCTGT GCAAGAACCT GATG

24

(2) INFORMATION FOR SEQ ID NO:20:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

- 152 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCAAAGGCA GGGCATACTT CCTG

24

(2) INFORMATION FOR SEQ ID NO:21:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGATCCTGA AGCTGCTCAG TTTG

24

25 (2) INFORMATION FOR SEQ ID NO:22:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

40 AGCGCGGCCG CGGGGACCAAC AATGAACAAAG TTG

33

(2) INFORMATION FOR SEQ ID NO:23:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

- 153 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

5 AGCTCTAGAA TTGTGAGGAA ACAGCTCAAT GGC

33

(2) INFORMATION FOR SEQ ID NO:24:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATAGCGGCCG CTGAGCCCAA ATCTTGTGAC AAAACTCAC

39

25 (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTAGAGTCG ACTTATCATT TACCCGGAGA CAGGGAGAGG CTCTT

45

40 (2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

- 154 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

5 (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

20 CCTCTGGCGC CGCTAACAG CTTATTTCA CGGATTGAAC CTG

43

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:29:

40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50

- 155 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCCGTAAGAA ACAGCCCCAGT GACC

24

5 (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

20 CCTCTGCGGC CGCTGTTGCA TTTCTTTCT G

31

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: protein

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His
1 5 10 15

40 Gln Leu Leu

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

- 156 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:33:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTCTGCGGC CGCACACACG TTGTCAATGTG TTGC

34

25 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

40 TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:35:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCTCTGCGGC CGCCTTTGC GTGGCTTCTC TGTT

34

(2) INFORMATION FOR SEQ ID NO:36:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTCTGAGCT CAAGCTTGTT TTCCGGGGAC CACAATG

37

25 (2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

40 CCTCTGCGGC CGCTAACGAG CTTATTTTA CTGAATGG

38

(2) INFORMATION FOR SEQ ID NO:38:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCTCTGAGCT CAAGCTTGGT TTCCSGGGAC CACAAATG

37

(2) INFORMATION FOR SEQ ID NO:39:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCTCTGCGGC CGCCAGGGTA ACATCTATTTC CAC

33

25 (2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

40 CCGAAGCTTC CACCATGAAC AAGTGGCTGT GCTGC

35

(2) INFORMATION FOR SEQ ID NO:41:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

5 CCTCTGTCGA CTATTATAAG CAGCTTATTT TCACGGATTG

40

(2) INFORMATION FOR SEQ ID NO:42:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCCCTTGCCC TGACCACTCT T

21

25 (2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCTCTGTCGA CTTAACACAC GTTGTCAATGT GTTGC

35

40 (2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

5 TCCCTTGCCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCTCTGTCGA CTTACTTTG CGTGGCTTCT CTGTT

35

(2) INFORMATION FOR SEQ ID NO:46:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1537 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTGAAGAGCGG	TGAAGAGCGG	TTCCCTCCTT	CAGCAAAAAA	CCCCTCAAGA	CCC GTTTAGA	60
40 GGCCCCAAGG	GGTTATGCTA	GTATTGCTC	AGCGGTGGCA	GCAGCCA ACT	CAGCTTCC TT	120
TCGGGCTTTC	TTCTTCTTCT	TCTTCTTCC	GCGGATCCTC	GAGTAAGCTT	CCATGGTACC	180
CTGCAGGTCTG	ACACTAGTGA	GCTCGAAC TC	CAACGGTTA	ACCATAATGTT	ATTCCCTC TT	240
45 TAATTAGTTA	AAACAAAATCT	AGAATCAAAT	CGATTAATCG	ACTATAACAA	ACC ATTTTCT	300
TGCGTAAACC	TGTACGATCC	TACAGGTACT	TATGTTAAC	AATTGTATT T	CAAGCGATAT	360
50 AATAGTGTGA	CAAAAATCCA	ATT TATTAGA	ATCAAATGTC	AATCTATTAC	CGTTTTAATG	420
ATATATAACA	CGCAAAACTT	GCGACAAACA	ATAGGTAAGG	ATAAAGAGAT	GGGTATGAAA	480

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	GACATAAAATG CCGACGACAC TTACAGAATA ATTAATAAAA TTAAGCCTG TAGAAGCAAT	540
5	AATGATATTA ATCAATGCTT ATCTGATATG ACTAAAATGG TACATTGTGA ATATTATTTA	600
	CTCGCGATCA TTTATCCTCA TTCTATGGTT AAATCTGATA TTTCAATTCT GGATAATTAC	660
	CCTAAAAAAT GGAGGCAATA TTATGATGAC GCTAATTAA TAAAATATGA TCCTATAGTA	720
10	GATTATTCTA ACTCCAATCA TTCACCGATT AATTGGAAATA TATTTGAAAA CAATGCTGTA	780
	AATAAAAAAT CTCCAAATGT AATTAAAGAA GCGAAATCAT CAGGTCTTAT CACTGGGTTT	840
15	AGTTTCCCTA TTCATACTGC TAATAATGGC TTCGGAATGC TTAGTTTGC ACATTCAAGAG	900
	AAAGACAACAT ATATAGATAG TTTATTTTA CATGCGTGTG TGAAACATACC ATTAATTGTT	960
	CCTTCTCTAG TTGATAATTA TCGAAAAATA AATATAGCAA ATAATAAACAT AACAGAACGAT	1020
20	TTAACCAAAA GAGAAAAAGA ATGTTTAGCG TGGGCATGCG AAGGAAAAAG CTCTTGGGAT	1080
	ATTTCAAAAA TATTAGGCTG TAGTAAGCGC ACGGTCACTT TCCATTAAAC CAATGCCAA	1140
25	ATGAAACTCA ATACAACAAA CCGCTGCCAA AGTATTCTA AAGCAATTAA AACAGGAGCA	1200
	ATTGATTGCC CATACTTTAA AAGTTAAGTA CGACGTCCAT ATTTGAATGT ATTAGAAAA	1260
	ATAAACAAAA GAGTTGTAG AACGCAAAA AGGCCATCCG TCAGGATGGC CTTCTGCTTA	1320
30	ATTTGATGCC TGGCAGTTA TGGCGGGCGT CCTGCCCGCC ACCCTCCGGG CGTTGCTTC	1380
	GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCTACTCA GGAGAGCGTT CACCGACAAA	1440
35	CAACAGATAAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT TTCGTTTAT TTGATGCCCTG	1500
	GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATAC	1537

(2) INFORMATION FOR SEQ ID NO:47:

- 40 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- 45 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

5 (2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

20 CGATTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAAATTC GGTAC

55

(2) INFORMATION FOR SEQ ID NO:49:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGAATTCCAA CGCGTTAACC ATAIGTTATT CCTCCTCTA GAATCAAAT

49

40 (2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1546 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

	GGCTAACGTA TGCATGGTCT CCCCAGCGA GAGTAGGGAA CTGCCAGGCA TCATAAATAAA	60
5	CGAAAGGCTC AGTCGAAAGA CTGGGCCTT CGTTTACT GTTGTGTC GGTGAACGCT	120
	CTCCTGAGTA GGACAAATCC CCCGGGAGCG GATTTGAAACG TTGCGAAGCA ACGGCCCGA	180
10	GGGTGGCGGG CAGGACGCC CCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGCCATC	240
	CTGACGGATG GCCTTTTGC GTTCTACAA ACTCTTTGT TTATTTTCT AAATACATTC	309
	AAATATGGAC GTCGTACTTA ACTTTAAAG TATGGGCAAT CAATTGCTCC TGTTAAAATT	360
15	GCTTAGAAA TACTTGGCA GCGGTTGTT GTATTGAGTT TCATTGCGC ATTGGTTAAA	420
	TGGAAAGTGA CCGTGCCTT ACTACAGCCT AATATTTTG AAATATCCCA AGAGCTTTT	480
20	CCTTCGCATG CCCACGCTAA ACATTCTTT TCTCTTTGG TAAATCGTT GTTGATTTA	540
	TTATTTGCTA TATTTATTT TCGATAATTA TCAACTAGAG AAGGAACAAT TAATGGTATG	600
	TTCATACACG CATGAAAAAA TAAACTATCT ATATAGTTGT CTTCTCTGA ATGTGAAAA	660
25	CTAACGCATTC CGAAGCCATT ATTACAGTA TGAATAGGGAA AACTAAACCC AGTGATAAGA	720
	CCTGATGATT TCGCTCTT AATTACATTT GGAGATTTT TATTACAGC ATTGTTTCA	780
30	AATATATTCC AATTAATCGG TGAATGATTG GAGTTAGAAT AATCTACTAT AGGATCATAT	840
	TTTATTAAAT TAGCGTCATC ATAATATTGC CTCCATTTT TAGGTAATT ATCCAGAATT	900
	GAAATATCAG ATTTAACCAT AGAATGAGGA TAAATGATCG CGAGTAAATA ATATTCACAA	960
35	TGTACCATTT TAGTCATATC AGATAAGCAT TGATTAATAI CATTATTGCT TCTACAGGCT	1020
	TTAATTTAT TAATTATTCT GTAAGTGTG TCGGCATTAA TGTCTTCAT ACCCATCTCT	1080
40	TTATCCTAC CTATTGTTG TCGCAAGTTT TCGGTGTTAT ATATCATTAA AACGGTAATA	1140
	GATTGACATT TGATTCTAAT AAATTGGATT TTTGTACAC TATTATATCG CTTGAAATAC	1200
	AATTGTTAA CATAAGTACC TGTAGGATCG TACAGGTTA CGCAAGAAAA TGGTTGTTA	1260
45	TAGTCGATTA ATCGATTGTA TTCTAGATTT GTTTAACTA ATTAAAGGAG GAATAACATA	1320
	TGGTTAACGC GTTGGAAATC GAGCTCACTA GTGTGACCT GCAGGGTACC ATGGAAGCTT	1380
	ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC CCGAAAGGAA GCTGAGTTGG	1440
50	CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG GGCCTCTAAA CGGGTCTTGA	1500

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GGGGTTTTT GCTGAAAGGA GGAACCGCTC TTCA CGCTCT TCACGC

1546

5 (2) INFORMATION FOR SEQ ID NO:51:

10

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

20

TATGAAACAT CATCACCATC ACCATCATGC TAGCGTTAAC GCGTTGG

47

25

(2) INFORMATION FOR SEQ ID NO:52:

30

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AATTCCAACG CGTTAACGCT AGCATGATGG TGATGGTGAT GATGTTCA

49

40

(2) INFORMATION FOR SEQ ID NO:53:-

45

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 141 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

5	CTAATTCCGC TCTCACCTAC CAAACAATGC CCCCCTGCAA AAAATAAAATT CATATAAAA	60
	ACATACAGAT AACCATCTGC GGTGATAAAAT TATCTCTGGC GGTGTTGACA TAAATACCAAC	120
	TGGCGGTGAT ACTGAGGCACA T	141

10 (2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 147 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

25	CGATGTGCTC AGTATCACCG CCAGTGGTAT TTATGTCAAC ACCGCCAGAG ATAATTATC	60
	ACCGCAGATG GTTATCTGTA TGTTTTTAT ATGAATTTAT TTTTGAGG GGGGCATTGT	120
30	TTGGTAGGTG AGAGCGGAAT TAGACGT	147

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 55 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

45	CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAAATTC GGTAC	55
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50

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(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

15

CGAATTCCAA CGCGTTAACCG ATATGTTATT CCTCCTTCTA GAATCAAAT

49

(2) INFORMATION FOR SEQ ID NO:57:

20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 668 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

35

GTGAAGAGCGG TGAAGAGCGG TTCCCTCTTT CAGCAAAAAA CCCCTCAAGA CCCGTTAGA

60

40

GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAAGT CAGCTTCCTT

120

TCGGGCTTTC TTCTTCTTCT TCTTCTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC

180

CTGCAGGTCTG ACACTAGTGA GCTCGAATTG CAACCGTTA ACCATATGTT ATTCTTCCTT

240

TAATTAGTTA ACTCAAATCT AGAATCAAAT CGATAAAATTG TGAGCGCTCA CAATTGAGAA

300

TATTAATCAA GAATTTAGC ATTTGTCAA TGAAATTTTT AAAAATTATG AGACGTCCAT

360

45

ATTTGAATGT ATTTAGAAAA ATAAACAAAA GAGTTTGTAG AAACGCAAAA AGGCCATCCG

420

TCAGGATGGC CTTCTGCTTA ATTTGATGCC TGGCAGTTA TGGCGGGCGT CCTGCCCGCC

480

50

ACCCCTCCGGG CCGTTGCTTC GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCTACTCA

540

GGAGAGCGTT CACCGACAAA CAACAGATAA AACGAAAGGC CCAGTC TC GACTGAGCCT

600

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TTCGTTTAT TTGATGCCTG GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATAACGTT 660

ACGCACGT 668

5

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 726 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

20 GCGTAACGTA TGCATGGTCT CCCCATGCCA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA 60

CGAAAAGGCTC AGTCGAAAGA CTGGGCCCTT CGTTTTATCT GTTGTTTGTG GGTGAACGCT 120

25 CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTGAACG TTGCGAAGCA ACGGCCCGGA 180

GGGTGGCGGG CAGGACGCCGCC GCCATAAAACT GCCAGGCATC AAATTAAGCA GAAGGGGCCT 240

30 CCCACCGCCC GTCCTGCCGG CGGTATTTGA CGGTCCGTAG TTTAATTCTGT CTTCGCCATC 300

CTGACGGATG GCCTTTTGTC GTTCTACAA ACTCTTTGT TTATTTTCT AAATACATTC 360

AAATATGGAC GTCTCATAAT TTTTAAAAAA TTCATTTGAC AAATGCTAAA ATTCTTGATT 420

35 AATATTCTCA ATTGTGAGCG CTCACAATTT ATCGATTGTA TTCTAGATTT GTTTAACTA 480

ATTAAAGGAG GAATAACATA TGGTTAACGC GTTGGAAATTG GAGCTCACTA GTGTCGACCT 540

40 GCAGGGTACC ATGGAAGCTT ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC 600

CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG 660

GGCCTCTAAA CGGGCTTGA GGGGTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT 720

45 TCACGC 726

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(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TACCGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT GGAC

44

(2) INFORMATION FOR SEQ ID NO:60:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GTCCTCCTGG TACCTACCTA AAACAAAC

27

35 (2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTACCCCCGG

60

50 GACATTTATC ACACAGCAGC TGATGAGAAG TTTCTTCATC CA

102

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(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

15 Met Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Cys Pro Pro
1 5 10 15

Gly Thr Tyr

20

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATGGAAACT TTTCTTCAA AATATCTTCA TTATGATGAA GAAACTTCTC ATCAGCTGCT 60
GTGTGATAAA TGTCGGCGG GTAC 84

40

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

5 CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCATAA TGAAGATATT
TTGGAGGAAA AGTTTCCA

60

78

(2) INFORMATION FOR SEQ ID NO:65:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 44 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTCACGGATT GAAC

44

25 (2) INFORMATION FOR SEQ ID NO:66:

- 30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 38 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
35 (ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

GTGCTCCTGG TACCTACCTA AAACAGCACT GCACAGTG

38

45 (2) INFORMATION FOR SEQ ID NO:67:

- 45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 84 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
50 (ii) MOLECULE TYPE: cDNA

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATGGAAACT CTGCCTCCAA AATACCTGCA TTACGATCCG GAAACTGGTC ATCAGCTGCT 60
GTGTGATAAA TGTGCTCCGG GTAC 84

10 (2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 79 base pairs
- (B) TYPE: nucleic acid
- 15 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

25 CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT 60
TTGGAGGCAG AGTTTCCA 78

30 (2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- 35 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TATGGACCCA GAAACTGGTC ATCAGCTGCT GTGTGATAAA TGTGCTCCGG GTAC 54

45 (2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- 50 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC TGGGTCCA

48

10

(2) INFORMATION FOR SEQ ID NO:71:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 87 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

TATGAAAGAA ACTCTGCCTC CAAAATACCT GCATTACGAT CCGGAAACTG GTCATCAGCT

60

GCTGTGTGAT AAATGTGCTC CGGGTAC

87

30

(2) INFORMATION FOR SEQ ID NO:72:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 81 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT

60

TTGGAGGCAG AGTTTCTTTC A

81

50

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(2) INFORMATION FOR SEQ ID NO:73:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 71 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GTTCTCCTCA TATGAAACAT CATCACCATC ACCATCATGA AACTCTGCCT CCAAAATACC

60

TGGATTACGA T

71

20 (2) INFORMATION FOR SEQ ID NO:74:

- 25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 43 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

35 GTTCTCCTCA TATGAAAGAA ACTCTGCCTC CAAAATACCT GCA

43

40 (2) INFORMATION FOR SEQ ID NO:75:

- 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 76 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TACGGCACTGG ATCCTTAATG ATGGTGATGG TGATGATGTA AGCAGCTTAT TTTCACGGAT 60

5 TGAACCTGAT TCCCTA 76

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GTTCTCCTCA TATGAAATAAC CTGCATTACG ATCCGGAAAC TGGTCAT 47

(2) INFORMATION FOR SEQ ID NO:77:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GTTCTCCTAT TAATGAAATA TCTTCATTAT GATGAAGAAA CTT

43

40 (2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

5 TACGGCACTGG ATCCTTATAA GCAGCTTATT TTACTGATT 40

(2) INFORMATION FOR SEQ ID NO:79:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

GTTCTCCTCA TATGGAAACT CTGCCTCCAA AATACCTGCA 40

25 (2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TACGGCACTGG ATCCTTATGT TGCATTCCT TTCTGAATTA GCA 43

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

5 CCGGAAACAG ATAATGAG

13

(2) INFORMATION FOR SEQ ID NO:82:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xii) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GATCCTCATT ATCTGTTT

13

25 (2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCGGAAACAG AGAAGCCACG CAAAAGTAAG

30

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

5 GATCCTTACT TTTGGGTGGC TTCTCTGTTT

30

(2) INFORMATION FOR SEQ ID NO:85:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATGTTAACAG AG

12

25 (2) INFORMATION FOR SEQ ID NO:86:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

40 GATCCTCAATT AACAA

14

(2) INFORMATION FOR SEQ ID NO:87:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

5 TATGTTCCGG AAACAGTTAA G

21

(2) INFORMATION FOR SEQ ID NO:88:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

25 GATCCTTAAC TGTTTCCGGA ACA

23

(2) INFORMATION FOR SEQ ID NO:89:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

TATGTTCCGG AAACAGTGAA TCAACTCAA AATAAG

36

(2) INFORMATION FOR SEQ ID NO:90:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GATCCTTATT TTGAGTTGA TTCACTGTTT CCGGAACA

38

10 (2) INFORMATION FOR SEQ ID NO:91:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

25 CTAGCGACGA CGACGACAAA GAAACTCTGC CTCCAAAATA CCTGCATTAC GATCCGGAAA

60

CTGGTCATCA GCTGCTGTGT GATAAAATGTG CTCCGGGTAC

100

30 (2) INFORMATION FOR SEQ ID NO:92:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

45 CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT

60

TTGGAGGCAG AGTTTCTTTG TCGTCGTCGT CG

92

50

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(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ACAAACACAAA TCGATTTGAT ACTAGA

26

(2) INFORMATION FOR SEQ ID NO:94:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TTTGTTTTAA CTAATTAAAG GAGGAATAAA ATATGAGAGG ATCGCATCAC

50

35 (2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

50 CATCACCATC ACGAACCTT CCCGCCAAA TACCTGCACT ACGACGAAGA

50

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(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

AACCTCCCAC CAGCTGCTGT GCGACAAATG CCCGCCGGGT ACCCAAACA

49

(2) INFORMATION FOR SEQ ID NO:97:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

TGTTTGGGTA CCCGGCGGGC ATTTGT

26

35 (2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

50 CGCACAGCAG CTGGTGGGAG GTTTCTTCGT CGTAGTGCAG GTATTCGGC

50

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(2) INFORMATION FOR SEQ ID NO:99:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GGGAAGGTTT CGTGATGGTG ATGGTGATGC GATCCTCTCA TATTTTATT

49

(2) INFORMATION FOR SEQ ID NO:100:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

CCTCCCTTTAA TTAGTTAAA CAAATCTAGT ATCAAATCGA TTGTGTTTGT

50

(2) INFORMATION FOR SEQ ID NO:101:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

50 ACAAACACAA TCGATTGAT ACTAGATTG TTTTAACCAA TTAAAGGAGG AATAAAATG

59

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(2) INFORMATION FOR SEQ ID NO:102:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 48 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

16 CTAATTAAG GAGGAATAAA ATGAAAGAAA CTTTCCTCC AAAATATC

48

(2) INFORMATION FOR SEQ ID NO:103:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: cDNA

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

31 TGTGGGGTA CCCGGCGGAC ATTTATCACA C

35 (2) INFORMATION FOR SEQ ID NO:104:

- 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 59 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

51 ACAAACACAA TCGATTTGAT ACTAGATTTG TTTAACTAA TTAAAGGAGG AATAAAATG

59

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(2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 54 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

15 CTAATTAAG GAGGAATAAA ATGAAAAAAA AAGAAACTTT TCCTCCAAAA TATC

54

(2) INFORMATION FOR SEQ ID NO:106:

20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C

31

35 (2) INFORMATION FOR SEQ ID NO:107:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

50 CAGCCCGGGT AAAATGGAAA CGTTTCCTCC AAAATATCTT CATT

44

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(2) INFORMATION FOR SEQ ID NO:108:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

CGTTTCCATT TTACCCGGGC TGAGCGAGAG GCTCTTCTGC GTGT

44

(2) INFORMATION FOR SEQ ID NO:109:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

CGCTCAGCCC GGGTAAAATG GAAACGTTGC CTCCAAAATA CCTGC

45

35 (2) INFORMATION FOR SEQ ID NO:110:

- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

50 CCATTTACC CGGGCTGAGC GAGAGGGCTCT TCTGCGTGT

39

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(2) INFORMATION FOR SEQ ID NO:111:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

GAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

(2) INFORMATION FOR SEQ ID NO:112:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

CAGCTGCAGC TAAGCAGCTT ATTTTCACGG ATTG

34

35 (2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

50 AAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

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(2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

15

CAGCTGCAGC TAAGCAGCTT ATTTTACTG ATTGG

35

(2) INFORMATION FOR SEQ ID NO:115:

20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

CTAGAAGGAG GAATAACATA TGGAAACTTT TGCTCCAAAA TATCTTCATT ATGATGAAGA

60

35

AACTAGTCAT CAGCTGCTGT GTGATAAAATG TCCGCCGGGT AC

102

(2) INFORMATION FOR SEQ ID NO:116:

40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 94 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GACTAGTTTC TTCATCATAA TGAAGATATT
 5 TTGGAGCAAA AGTTTCCATA TGTTATTCCCT CCTT 60
 94

(2) INFORMATION FOR SEQ ID NO:117:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 62 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

CTAGAAGGAG GAATAACATA TGGAAAACTTT TCCTGCTAAA TATCTTCATT ATGATGAAGA 50
 AA

25 (2) INFORMATION FOR SEQ ID NO:118:
 62

- 30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 62 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

CTAGTTTCTT CATCATAATG AAGATATTAA GCAGGAAAAG TTTCCATATG TTATTCCTCC 60
 TT

45 (2) INFORMATION FOR SEQ ID NO:119:

- 50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 91 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

10	Tyr His Tyr Tyr Asp Gln Asn Gly Arg Met Cys Glu Glu Cys His Met 1 5 10 15
	Cys Gln Pro Gly His Phe Leu Val Lys His Cys Lys Gln Pro Lys Arg 20 25 30
15	Asp Thr Val Cys His Lys Pro Cys Glu Pro Gly Val Thr Tyr Thr Asp 35 40 45
	Asp Trp His 50

20 (2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2432 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: sing

1

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 124-1326

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

ATCAAAGGCA GGGCATACTT CCTGTTGCCA AGACCTTATA TAAAACGTCA TGTTCGCCCTG
 40 GGCAGCAGAG AAGCACCTAG CACTGGCCCA GCGGCTGCCG CCTGAGGTTT CCAGAGGACC
 ACA ATG AAC AAG TGG CTG TGC TGT GCA CTC CTG GTG TTC TTG GAC ATC
 Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile
 1 5 10 15
 45 ATT GAA TGG ACA ACC CAG GAA ACC TTT CCT CCA AAA TAC TTG CAT TAT
 Ile Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr
 20 25 30

50

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	GAC CCA GAA ACC GGA CGT CAG CTC TTG TGT GAC AAA TGT GCT CCT GGC Asp Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly 35	40	45	264
5	ACC TAC CTA AAA CAG CAC TGC ACA GTC AGG AGG AAG ACA CTG TGT GTC Thr Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val 50	55	60	312
10	CCT TGC CCT GAC TAC TCT TAT ACA GAC AGC TGG CAC ACG AGT GAT GAA Pro Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu 65	70	75	360
15	TGC GTG TAC TGC AGC CCC GTG TGC AAG GAA CTG CAG ACC GTG AAA CAG Cys Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln 80	85	90	408
20	GAG TGC AAC CGC ACC CAC AAC CGA GTG TGC GAA TGT GAG GAA GGG CGC Glu Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg 100	105	110	456
25	TAC CTG GAG CTC GAA TTC TGC TTG AAG CAC CGG AGC TGT CCC CCA GGC Tyr Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly 115	120	125	504
30	TTG GGT GTG CTG CAG GCT GGG ACC CCA GAG CGA AAC ACG GTT TGC AAA Leu Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys 130	135	140	552
35	AGA TGT CCG GAT GGG TTC TTC TCA GGT GAG ACG TCA TCG AAA GCA CCC Arg Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro 145	150	155	600
40	TGT AGG AAA CAC ACC AAC TGC AGC TCA CTT GGC CTC CTG CTA ATT CAG Cys Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln 160	165	170	648
45	AAA GGA AAT GCA ACA CAT GAC AAT GTA TGT TCC GGA AAC AGA GAA GCA Lys Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala 180	185	190	696
50	ACT CAA AAT TGT GGA ATA GAT GTC ACC CTG TGC GAA GAG GCA TTC TTC Thr Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe 195	200	205	744
	AGG TTT GCT GTG CCT ACC AAG ATT ATA CCG AAT TGG CTG AGT GTT CTG Arg Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu 210	215	220	792
	GTG GAC AGT TTG CCT GGG ACC AAA GTG AAT GCA GAG AGT GTA GAG AGG Val Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg 225	230	235	840

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	ATA AAA CGG AGA CAC AGC TCG CAA GAG CAA ACT TTC CAG CTA CTT AAG Ile Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys 240 245 250 255	938
5	CTG TGG AAG CAT CAA AAC AGA GAC CAG GAA ATG GTG AAG AAG ATC ATC Leu Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile 260 265 270	936
10	CAA GAC ATT GAC CTC TGT GAA AGC AGT GTG CAA CGG CAT ATC GGC CAC Gln Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His 275 280 285	984
15	GCG AAC CTC ACC ACA GAG CAG CTC CGC ATC TTG ATG GAG AGC TTG CCT Ala Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro 290 295 300	1032
20	GGG AAG AAG ATC AGC CCA GAC GAG ATT GAG AGA ACG AGA AAG ACC TGC Gly Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys 305 310 315	1080
25	AAA CCC AGC GAG CAG CTC CTG AAG CTA CTG AGC TTG TGG AGG ATC AAA Lys Pro Ser Glu Gln Leu Leu Lys Leu Ser Leu Trp Arg Ile Lys 320 325 330 335	1128
30	AAT GGA GAC CAA GAC ACC TTG AAG GGC CTG ATG TAC GCA CTC AAG CAC Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His 340 345 350	1176
35	TTG AAA GCA TAC CAC TTT CCC AAA ACC GTC ACC CAC AGT CTG AGG AAG Leu Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys 355 360 365	1224
40	ACC ATC AGG TTC TTG CAC AGC TTC ACC ATG TAC CGA TTG TAT CAG AAA Thr Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys 370 375 380	1272
45	CTC TTT CTA GAA ATG ATA GGG AAT CAG GTT CAA TCA GTG AAG ATA AGC Leu Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser 385 390 395	1320
50	TGC TTA TAGTTAGGAA TGGTCACTGG GCTGTTCTT CAGGATGGC CAACACTGAT Cys Leu 400	1376
	GGAGCAGATG GCTGCTTCTC CGGCTCTTGA AATGGCAGTT GATTCCCTTC TCATCAGTTG GTGGGAATGA AGATCCTCCA GCCAACACA CACACTGGGG AGTCTGAGTC AGGAGAGTGA	1436
	GGCAGGGCTAT TTGATAATTG TCCAAAGCTG CCAGGTGTAC ACCTAGAAAG TCAAGCACCC	1496
		1556

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TGAGAAAGAG GATATTTTA TAAACCTCAAA CATAGGCCCTT TTCCCTTCCTC CCCTTATGGA	1616
TGAGTACTCA GAAGGCTTCT ACTATCTTCT GTGTCACTCCC TAGATGAAGG CCTCTTTTAT	1676
5 TTATTTTT ATTCTTTTT CGGGAGCTGG GGACCGAACCC CAGGGCCTTG CGCTTGCGAG	1736
GCAAGTGCTC TACCACTGAG CTAAATCTCC AACCCCTGAA GGCCCTTTTC TTTCTGCCTC	1796
10 TGATAGTCTA TGACATTCTT TTTCTACAA TTCGTATCAG GTGCACGAGC CTTATCCCCT	1856
TTGTAGGTTT CTAGGCAAGT TGACCGTTAG CTATTTTCC CTCTGAAGAT TTGATTGAG	1916
TTGCAGACTT GGCTAGACAA GCAGGGTAG GTTATGGTAG TTTATTAAC AGACTGCCAC	1976
15 CAGGAGTCCA GTGTTCTTG TTCCCTGTG GTTGTACCTA AGCTGACTCC AAGTACATT	2036
AGTAIGAAAA ATAATCAACA AATTTTATTCTT CTTCTATCAA CATTGGCTAG CTTTGTTC	2096
20 GGGCACTAA AGAAAACTACT ATATGGAGAA AGAATTGATA TTGCCCCCAA CGTTCAACAA	2156
CCCAATAGTT TATCCAGCTG TCATGCCTGG TTCACTGTCT ACTGACTATG CGCCCTCTTA	2216
TTACTGCATG CAGTAATTCA ACTGGAAATA GTAATAATAA TAATAGAAAT AAAATCTAGA	2276
25 CTCCATTGGA TCTCTCTGAA TATGGGAATA TCTAACTTAA GAAGCTTGA GATTTCAGTT	2336
GTGTTAAAGG CTTTATTAA AAAGCTGATG CTCTCTGTGAA AAAGTTACTA ATATATCTGT	2396
30 AAGACTATTAA CAGTATTGCT ATTTATATCC ATCCAG	2432

(2) INFORMATION FOR SEQ ID NO:121:

35	(i) SEQUENCE CHARACTERISTICS:
	(A) LENGTH: 401 amino acids
	(B) TYPE: amino acid
	(D) TOPOLOGY: linear
40	(ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile			
1	5	10	15
50 Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp			
20	25	30	

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	Pro	Glu	Thr	Gly	Arg	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	Thr
							35			40						45
5	Tyr	Leu	Lys	Gln	His	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro
							50			55						60
	Cys	Pro	Asp	Tyr	Ser	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys
							65			70						80
10	Val	Tyr	Cys	Ser	Pro	Val	Cys	Lys	Glu	Leu	Gln	Thr	Val	Lys	Gln	Glu
							85			90						95
	Cys	Asn	Arg	Thr	His	Asn	Arg	Val	Cys	Glu	Cys	Glu	Glu	Gly	Arg	Tyr
15							100				105					110
	Leu	Glu	Leu	Glu	Phe	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Leu
							115			120						125
20	Gly	Val	Leu	Gln	Ala	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg
							130			135						140
	Cys	Pro	Asp	Gly	Phe	Phe	Ser	Gly	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys
							145			150						160
25	Arg	Lys	His	Thr	Asn	Cys	Ser	Ser	Leu	Gly	Leu	Leu	Ile	Gln	Lys	
							165			170						175
	Gly	Asn	Ala	Thr	His	Asp	Asn	Val	Cys	Ser	Gly	Asn	Arg	Glu	Ala	Thr
30							180			185						190
	Gln	Asn	Cys	Gly	Ile	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg
							195			200						205
35	Phe	Ala	Val	Pro	Thr	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val
							210			215						220
	Asp	Ser	Leu	Pro	Gly	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile
							225			230						240
40	Lys	Arg	Arg	His	Ser	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu
							245			250						255
	Trp	Lys	His	Gln	Asn	Arg	Asp	Gln	Glu	Met	Val	Lys	Ile	Ile	Gln	
45							260			265						270
	Asp	Ile	Asp	Leu	Cys	Glu	Ser	Ser	Val	Gln	Arg	His	Ile	Gly	His	Ala
							275			280						285
50	Asn	Leu	Thr	Thr	Glu	Gln	Leu	Arg	Ile	Leu	Met	Glu	Ser	Leu	Pro	Gly
							290			295						300

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Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys
 305 310 315 320

5 Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
 325 330 335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu
 10 340 345 350

Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr
 355 360 365

15 Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu
 370 375 380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
 385 390 395 400

20 Leu

(2) INFORMATION FOR SEQ ID NO:122:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1324 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 90..1292

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

CCTTATATAAA ACGTCATGAT TGCCTGGGCT GCAGAGACGC ACCTAGCACT GACCCAGCGG 60

CTGCCTCCTG AGGTTCCCCG AGGACCACCA ATG AAC AAG TGG CTG TGC TGC GCA 113

45 Met Asn Lys Trp Leu Cys Cys Ala
1 5

CTC CTG GTG CTC CTG GAC ATC ATT GAA TGG ACA ACC CAG GAA ACC CTT 161

Leu Leu Val Leu Leu Asp Ile Ile Glu Trp Thr Thr Gln Glu Thr Leu

50 10 15 20

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	CCT CCA AAG TAC TTG CAT TAT GAC CCA GAA ACT GGT CAT CAG CTC CTG Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His Gin Leu Leu 25 30 35 40	209
5	TGT GAC AAA TGT GCT CCT GGC ACC TAC CTA AAA CAG CAC TGC ACA GTG Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gin His Cys Thr Val 45 50 55	257
10	AGG AGG AAG ACA TTG TGT GTC CCT TGC CCT GAC CAC TCT TAT ACG GAC Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser Tyr Thr Asp 60 65 70	305
15	AGC TGG CAC ACC AGT GAT GAG TGT GTG TAT TGC AGC CCA GTG TGC AAG Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro Val Cys Lys 75 80 85	353
20	GAA CTG CAG TCC GTG AAG CAG GAG TGC AAC CGC ACC CAC AAC CGA GTG Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val 90 95 100	401
25	TGT GAG TGT GAG GAA GGG CGT TAC CTG GAG ATC GAA TTC TGC TTG AAG Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys 105 110 115 120	449
30	CAC CGG AGC TGT CCC CCG GGC TCC GGC GTG GTG CAA GCT GGA ACC CCA His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala Gly Thr Pro 125 130 135	497
35	GAG CGA AAC ACA GTT TGC AAA AAA TGT CCA GAT GGG TTC TTC TCA GGT Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe Ser Gly 140 145 150	545
40	GAG ACT TCA TCG AAA GCA CCC TGT ATA AAA CAC ACG AAC TGC AGC ACA Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn Cys Ser Thr 155 160 165	593
45	TTT GGC CTC CTG CTA ATT CAG AAA GGA AAT GCA ACA CAT GAC AAC GTG Phe Gly Leu Leu Ile Gln Lys Gly Asn Ala Thr His Asp Asn Val 170 175 180	641
50	TGT TCC GGA AAC AGA GAA GCC ACG CAA AAG TGT GGA ATA GAT GTC ACC Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile Asp Val Thr 185 190 195 200	689
	CTG TGT GAA GAG GCC TTC TTC AGG TTT GCT GTT CCT ACC AAG ATT ATA Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Ile Ile 205 210 215	737

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	CCA AAT TGG CTG AGT GTT TTG GTG GAC AGT TTG CCT GGG ACC AAA GTG Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly Thr Lys Val 220 225 230	735
5	AAT GCC GAG AGT GTA GAG AGG ATA AAA CGG AGA CAC AGC TCA CAA GAG Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser Ser Gln Glu 235 240 245	833
10	CAA ACC TTC CAG CTG CTG AAG CTG TGG AAA CAT CAA AAC AGA GAC CAG Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Arg Asp Gln 250 255 260	381
15	GAA ATG GTG AAG AAG ATC ATC CAA GAC ATT GAC CTC TGT GAA AGC AGC Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Ser Ser 265 270 275 280	929
20	GTG CAG CGG CAT CTC GGC CAC TCG AAC CTC ACC ACA GAG CAG CTT CTT Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu Gln Leu Leu 285 290 295	977
25	GCC TTG ATG GAG AGC CTG CCT GGG AAG AAG ATC AGC CCA GAA GAG ATT Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro Glu Glu Ile 300 305 310	1025
30	GAG AGA ACG AGA AAG ACC TGC AAA TCG AGC GAG CAG CTC CTG AAG CTA Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu Leu Lys Leu 315 320 325	1073
35	CTC AGT TTA TGG AGG ATC AAA AAT GGT GAC CAA GAC ACC TTG AAG GGC Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly 330 335 340	1121
40	CTG ATG TAT GCC CTC AAG CAC TTG AAA ACA TCC CAC TTT CCC AAA ACT Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe Pro Lys Thr 345 350 355 360	1169
45	GTC ACC CAC AGT CTG AGG AAG ACC ATG AGG TTC CTG CAC AGC TTC ACA Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His Ser Phe Thr 365 370 375	1217
50	ATG TAC AGA CTG TAT CAG AAG CTC TTT TTA GAA ATG ATA GGG AAT CAG Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn Gln 380 385 390	1265
	GCTGTTCTT CA	1324

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(2) INFORMATION FOR SEQ ID NO:123:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 401 amino acid
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

15 Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Leu Leu Asp Ile Ile
15 1 5 10 15

Glu Trp Thr Thr Gln Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp
20 25 30

20 Pro Glu Thr Gly His Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
35 40 45

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
50 55 60

25 Cys Pro Asp His Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
 65 70 75 80

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
 100 105 110

35 Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser
115 120 125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys
130 135 140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
145 150 155 160

45 Ile Lys His Thr Asn Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys
165 170 175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
180 185 190

50 Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
195 200 205

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	Phe	Ala	Val	Pro	Thr	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val
	210						215					220				
5	Asp	Ser	Leu	Pro	Gly	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile
	225						230				235				240	
	Lys	Arg	Arg	His	Ser	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu
10					245					250			255			
	Trp	Lys	His	Gln	Asn	Arg	Asp	Gln	Glu	Met	Val	Lys	Lys	Ile	Ile	Gln
					260				265			270				
15	Asp	Ile	Asp	Leu	Cys	Glu	Ser	Ser	Val	Gln	Arg	His	Leu	Gly	His	Ser
					275				280			285				
	Asn	Leu	Thr	Thr	Glu	Gln	Leu	Leu	Ala	Leu	Met	Glu	Ser	Leu	Pro	Gly
					290			295			300					
20	Lys	Lys	Ile	Ser	Pro	Glu	Glu	Ile	Glu	Arg	Thr	Arg	Lys	Thr	Cys	Lys
					305			310			315			320		
	Ser	Ser	Glu	Gln	Leu	Leu	Lys	Leu	Leu	Ser	Leu	Trp	Arg	Ile	Lys	Asn
25					325				330			335				
	Gly	Asp	Gln	Asp	Thr	Leu	Lys	Gly	Leu	Met	Tyr	Ala	Leu	Lys	His	Leu
					340				345			350				
30	Lys	Thr	Ser	His	Phe	Pro	Lys	Thr	Val	Thr	His	Ser	Leu	Arg	Lys	Thr
					355				360			365				
	Met	Arg	Phe	Leu	His	Ser	Phe	Thr	Met	Tyr	Arg	Leu	Tyr	Gln	Lys	Leu
					370			375			380					
35	Phe	Leu	Glu	Met	Ile	Gly	Asn	Gln	Val	Gln	Ser	Val	Lys	Ile	Ser	Cys
					385			390			395			400		
	Leu															
40	(2) INFORMATION FOR SEQ ID NO:124:															
	(i) SEQUENCE CHARACTERISTICS:															
45	(A) LENGTH: 1355 base pairs															
	(B) TYPE: nucleic acid															
	(C) STRANDEDNESS: single															
	(D) TOPOLOGY: linear															
50	(ii) MOLECULE TYPE: cDNA															

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(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 94..1296

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

	GTATATATAA CGTGATGAGC GTACGGGTGC GGAGACGCAC CGGAGCGCTC GCCCAGCCGC	60
10	CGCTCCAAGC CCCTGAGGTT TCCGGGGACC ACA ATG AAC AAG TTG CTG TGC TGC Met Asn Lys Leu Leu Cys Cys	114,
	1 5	
15	GCG CTC GTG TTT CTG GAC ATC TCC ATT AAG TGG ACC ACC CAG GAA ACG Ala Leu Val Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr 10 15 20	162
20	TTT CCT CCA AAG TAC CTT CAT TAT GAC GAA GAA ACC TCT CAT CAG CTG Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His Gln Leu 25 30 35	210
25	TTG TGT GAC AAA TGT CCT GGT ACC TAC CTA AAA CAA CAC TGT ACA Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His Cys Thr 40 45 50 55	258
30	GCA AAG TGG AAG ACC GTG TGC GCC CCT TGC CCT GAC CAC TAC TAC ACA Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr 60 65 70	306
35	GAC AGC TGG CAC ACC AGT GAC GAG TGT CTA TAC TGC AGC CCC GTG TGC Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys 75 80 85	354
40	AAG GAG CTG CAG TAC GTC AAG CAG GAG TGC AAT CGC ACC CAC AAC CGC Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg 90 95 100	402
45	GTG TGC GAA TGC AAG GAA GGG CGC TAC CTT GAG ATA GAG TTC TGC TTG Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu 105 110 115	450
50	AAA CAT AGG AGC TGC CCT CCT GGA TTT GGA GTG GTG CAA GCT GGA ACC Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr 120 125 130 135	498
	CCA GAG CGA AAT ACA GTT TGC AAA AGA TGT CCA GAT GGG TTC TTC TCA Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe Ser 140 145 150	546

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	AAT GAG ACG TCA TCT AAA GCA CCC TGT AGA AAA CAC ACA AAT TGC AGT Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn Cys Ser 155 160 165	594
5	GTC TTT GGT CTC CTG CTA ACT CAG AAA GGA AAT GCA ACA CAC GAC AAC Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His Asp Asn 170 175 180	642
10	ATA TGT TCC GGA AAC AGT GAA TCA ACT CAA AAA TGT GGA ATA GAT GTT Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile Asp Val 185 190 195	690
15	ACC CTG TGT GAG GAG GCA TTC TTC AGG TTT GCT GTT CCT ACA AAG TTT Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Phe 200 205 210 215	738
20	ACG CCT AAC TGG CTT AGT GTC TTG GTA GAC AAT TTG CCT GGC ACC AAA Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly Thr Lys 220 225 230	786
25	GTA AAC GCA GAG AGT GTA GAG AGG ATA AAA CGG CAA CAC AGC TCA CAA Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser Ser Gln 235 240 245	834
30	GAA CAG ACT TTC CAG CTG CTG AAG TTA TGG AAA CAT CAA AAC AAA GCC Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Lys Ala 250 255 260	882
35	CAA GAT ATA GTC AAG AAG ATC ATC CAA GAT ATT GAC CTC TGT GAA AAC Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Asn 265 270 275	930
40	AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC TTC GAG CAG CTT Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu Gln Leu 280 285 290 295	978
45	CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG GGA GCA GAA GAC Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp 300 305 310	1026
50	ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC CAG ATC CTG AAG Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys 315 320 325	1074
	CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA GAC ACC TTG AAG Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys 330 335 340	1122

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GGC CTA ATG CAC GCA CTA AAG CAC TCA AAG ACG TAC CAC TTT CCC AAA	1170
Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys	
345	350
	355
 5 ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC CTT CAC AGC TTC	1218
Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe	
360	365
	370
	375
 10 ACA ATG TAC AAA TTG TAT CAG AAG TTA TTT TTA GAA ATG ATA GGT AAC	1266
Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn	
380	385
	390
 15 CAG GTC CAA TCA GTA AAA ATA AGC TGC TTA TAACTGGAAA TGGCCATTGA	1316
Gln Val Gln Ser Val Lys Ile Ser Cys Leu	
395	400
 GCTGTTTCCT CACAATTGGC GAGATCCCAT GGATGATAA	1355

20 (2) INFORMATION FOR SEQ ID NO:125:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 401 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

30 Met Asn Lys Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile
 1 5 10 15

 35 Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
 20 25 30

 40 Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr
 35 40 45

 45 Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro
 50 55 60

 50 Cys Pro Asp His Tyr Tyr Asp Ser Trp His Thr Ser Asp Glu Cys
 65 70 75 80

 55 Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu
 85 90 95

 60 Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr
 100 105 110

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	Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe			
	115	120	125	
5	Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg			
	130	135	140	
	Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys			
	145	150	155	160
10	Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys			
	165	170	175	
	Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr			
15	180	185	190	
	Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg			
	195	200	205	
20	Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val			
	210	215	220	
	Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile			
	225	230	235	240
25	Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu			
	245	250	255	
	Trp Lys His Gln Asn Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln			
30	260	265	270	
	Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala			
	275	280	285	
35	Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly			
	290	295	300	
	Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys			
	305	310	315	320
40	Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn			
	325	330	335	
	Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser			
45	340	345	350	
	Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr			
	355	360	365	
50	Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu			
	370	375	380	

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Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
385 390 395 400

Leu

5

(2) INFORMATION FOR SEQ ID NO:126:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 139 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(iii) MOLECULES TYPE

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 100

Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys
1 5 10 15

25 Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
 20 25 30

Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala
35 40 45

Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys
50 55 60

Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn
85 90 95

40 Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His
100 105 110

Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly
115 120 125

Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys
130 135

50

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(2) INFORMATION FOR SEQ ID NO:127:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

20

(2) INFORMATION FOR SEQ ID NO:128:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 219 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: protein

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
1 5 10 15

40 Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
20 25 30

45 Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Val Glu Thr Gln Asn
35 40 45

Lys Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
50 55 60

50 Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro
65 70 75 80

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	Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
	85 90 95
5	Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly
	100 105 110
	Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg
	115 120 125
10	Cys Lys Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp
	130 135 140
	Pro Cys Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr
15	145 150 155 160
	Ser Asn Thr Lys Cys Lys Glu Glu Gly Ser Arg Ser Asn Leu Gly Trp
	165 170 175
20	Leu Cys Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg
	180 185 190
	Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly
	195 200 205
25	Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr
	210 215

(2) INFORMATION FOR SEQ ID NO:129:

30	(i) SEQUENCE CHARACTERISTICS:
	(A) LENGTH: 280 amino acids
	(B) TYPE: amino acid
	(C) STRANDEDNESS: single
35	(D) TOPOLOGY: linear
	(ii) MOLECULE TYPE: protein

40	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:
----	---

45	Met Gly Leu Ser Thr Val Pro Asp Leu Leu Pro Leu Val Leu Leu
	1 5 10 15
	Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
	20 25 30
50	His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
	35 40 45

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Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
5 65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
85 90 95

10 Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
115 120 125

15 Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
130 135 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
20 145 150 155 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
165 170 175

25 Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
180 185 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
30 195 200 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
210 215 220

35 Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
225 230 235 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
40 245 250 255

Gly Glu Leu Glu Gly Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
260 265 270

Phe Ser Pro Thr Pro Gly Phe Thr
45 275 280

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(2) INFORMATION FOR SEQ ID NO:130:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr Val Tyr Gly
1 5 10 15

Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly Gly His Asp
 20 25 30

Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro Gly Phe Tyr
35 40 45

25 Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys Ser Pro Cys
50 55 60

Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val
65 70 75 80

Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser Gln Pro Cys
85 90 95

35 Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly Asn Tyr Cys
100 105 110

Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro Gln Thr Lys
115 120 125

40 Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala Gly Asp Thr
130 135 140

Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser Leu Ser Pro
145 150 155 160

45 Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val Gly Phe Asn

Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Thr Ala Gly His Asn

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Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn Tyr Thr
195 200 205

(2) INFORMATION FOR SEQ ID NO:131:

5

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 227 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu
20 1 5 10 15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr
20 25 30

25 Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln
35 40 45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys
50 55 60

30 Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp
65 70 75 80

35 Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys
85 90 95

40 Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg
100 105 110

Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu
115 120 125

45 Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg
130 135 140

Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val
145 150 155 160

50 Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr
165 170 175

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Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly
180 185 190

5 Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser
195 200 205

Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser
10 210 215 220

Gln His Thr
225

(2) INFORMATION FOR SEQ ID NO:132:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 197 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

30 Met Val Ser Leu Pro Arg Leu Cys Ala Leu Trp Gly Cys Leu Leu Thr
1 5 10 15

Ala Val His Leu Gly Gln Cys Val Thr Cys Ser Asp Lys Gln Tyr Leu
20 25 30

35 His Asp Gly Gln Cys Cys Asp Leu Cys Gln Pro Gly Ser Arg Leu Thr
35 40 45

40 Ser His Cys Thr Ala Leu Glu Lys Thr Gln Cys His Pro Cys Asp Ser
50 55 60

Gly Glu Phe Ser Ala Gln Trp Asn Arg Glu Ile Arg Cys His Gln His
65 70 75 80

45 Arg His Cys Glu Pro Asn Gln Gly Leu Arg Val Lys Lys Glu Gly Thr
85 90 95

Ala Glu Ser Asp Thr Val Cys Thr Cys Lys Glu Gly Gln His Cys Thr
100 105 110

50 Ser Lys Asp Cys Glu Ala Cys Ala Gln His Thr Pro Cys Ile Pro Gly
115 120 125

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Phe Gly Val Met Glu Met Ala Thr Glu Thr Thr Asp Thr Val Cys His
 130 135 140

5 Pro Cys Pro Val Gly Phe Phe Ser Asn Gln Ser Ser Leu Phe Glu Lys
 145 150 155 160

Cys Tyr Pro Trp Thr Ser Cys Glu Asp Lys Asn Leu Glu Val Leu Gln
165 170 175

10 Lys Gly Thr Ser Gln Thr Asn Val Ile Cys Gly Leu Lys Ser Arg Met
180 185 190

Arg Ala Leu Leu Val
195

(2) INFORMATION FOR SEQ ID NO:133:

(1) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 208 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile
1 5 10 15

35 Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
20 25 30

40 Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
35 40 45

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
50 55 60

45 Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
65 70 75 80

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu
85 90 95

50

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	Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
	100 105 110
5	Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu
	115 120 125
	Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
	130 135 140
10	Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
	145 150 155 160
	Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Ile Gln Lys
15	165 170 175
	Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
	180 185 190
20	Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
	195 200 205

(2) INFORMATION FOR SEQ ID NO:134:

25	(i) SEQUENCE CHARACTERISTICS:
	(A) LENGTH: 224 amino acids
	(B) TYPE: amino acid
	(C) STRANDEDNESS: single
	(D) TOPOLOGY: linear.
30	(ii) MOLECULE TYPE: protein

35	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:
----	---

40	Met Gly Ala Gly Ala Thr Gly Arg Ala Met Asp Gly Pro Arg Leu Leu
	1 5 10 15
	Leu Leu Leu Leu Leu Gly Val Ser Leu Gly Gly Ala Lys Glu Ala Cys
	20 25 30
45	Pro Thr Gly Leu Tyr Thz His Ser Gly Glu Cys Cys Lys Ala Cys Asn
	35 40 45
	Leu Gly Glu Gly Val Ala Gln Pro Cys Gly Ala Asn Gln Thr Val Cys
50	50 55 60

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	Glu Pro Cys Leu Asp Ser Val Thr Phe Ser Asp Val Val Ser Ala Thr			
65	70	75	80	
	Glu Pro Cys Lys Pro Cys Thr Glu Cys Val Gly Leu Gln Ser Met Ser			
5	85	90	95	
	Ala Pro Cys Val Glu Ala Asp Asp Ala Val Cys Arg Cys Ala Tyr Gly			
	100	105	110	
10	Tyr Tyr Gln Asp Glu Thr Thr Gly Arg Cys Glu Ala Cys Arg Val Cys			
	115	120	125	
	Glu Ala Gly Ser Gly Leu Val Phe Ser Cys Gln Asp Lys Gln Asn Thr			
	130	135	140	
15	Val Cys Glu Glu Cys Pro Asp Gly Thr Tyr Ser Asp Glu Ala Asn His			
	145	150	155	160
20	Val Asp Pro Cys Leu Pro Cys Thr Val Cys Glu Asp Thr Glu Arg Gln			
	165	170	175	
	Leu Arg Glu Cys Thr Arg Trp Ala Asp Ala Glu Cys Glu Glu Ile Pro			
	180	185	190	
25	Gly Arg Trp Ile Thr Arg Ser Thr Pro Pro Glu Gly Ser Asp Ser Thr			
	195	200	205	
	Ala Pro Ser Thr Gln Glu Pro Glu Ala Pro Pro Glu Gln Asp Leu Ile			
	210	215	220	
30				

(2) INFORMATION FOR SEQ ID NO:135:

- 35 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 205 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- 40 (ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

	Met Tyr Val Trp Val Gln Gln Pro Thr Ala Phe Leu Leu Leu Gly Leu		
1	5	10	15
50	Ser Leu Gly Val Thr Val Lys Leu Asn Cys Val Lys Asp Thr Tyr Pro		
	20	25	30

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	Ser	Gly	His	Lys	Cys	Cys	Arg	Glu	Cys	Gln	Pro	Gly	His	Gly	Met	Val
				35				40						45		
5	Ser	Arg	Cys	Asp	His	Thr	Arg	Asp	Thr	Val	Cys	His	Pro	Cys	Glu	Pro
		50				55				60						
	Gly	Phe	Tyr	Asn	Glu	Ala	Val	Asn	Tyr	Asp	Thr	Cys	Lys	Gln	Cys	Thr
		65				70			75			80				
10	Gln	Cys	Asn	His	Arg	Ser	Gly	Ser	Glu	Leu	Lys	Gln	Asn	Cys	Thr	Pro
					85				90			95				
15	Thr	Glu	Asp	Thr	Val	Cys	Gln	Cys	Arg	Pro	Gly	Thr	Gln	Pro	Arg	Gln
					100				105			110				
	Asp	Ser	Ser	His	Lys	Leu	Gly	Val	Asp	Cys	Val	Pro	Cys	Pro	Pro	Gly
					115			120			125					
20	His	Phe	Ser	Pro	Gly	Ser	Asn	Gln	Ala	Cys	Lys	Pro	Trp	Thr	Asn	Cys
					130			135			140					
	Thr	Leu	Ser	Gly	Lys	Gln	Ile	Arg	His	Pro	Ala	Ser	Asn	Ser	Leu	Asp
		145				150			155			160				
25	Thr	Val	Cys	Glu	Asp	Arg	Ser	Leu	Leu	Ala	Thr	Leu	Leu	Trp	Glu	Thr
					165			170			175					
30	Gln	Arg	Thr	Thr	Phe	Arg	Pro	Thr	Thr	Val	Pro	Ser	Thr	Thr	Val	Trp
					180			185			190					
	Pro	Arg	Thr	Ser	Gln	Leu	Pro	Ser	Thr	Pro	Thr	Leu	Val			
					195			200			205					

35 (2) INFORMATION FOR SEQ ID NO:136:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 191 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: protein

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

50	Met	Gly	Asn	Asn	Cys	Tyr	Asn	Val	Val	Val	Ile	Val	Leu	Leu	Leu	Val
	1				5				10				15			

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	Gly	Cys	Glu	Lys	Val	Gly	Ala	Val	Gln	Asn	Ser	Cys	Asp	Asn	Cys	Gln
				20					25						30	
5	Pro	Gly	Thr	Phe	Cys	Arg	Lys	Tyr	Asn	Pro	Val	Cys	Lys	Ser	Cys	Pro
				35					40					45		
10	Pro	Ser	Thr	Phe	Ser	Ser	Ile	Gly	Gly	Gln	Pro	Asn	Cys	Asn	Ile	Cys
				50					55				60			
15	Arg	Val	Cys	Ala	Gly	Tyr	Phe	Arg	Phe	Lys	Lys	Phe	Cys	Ser	Ser	Thr
				65					70				75			80
20	His	Asn	Ala	Glu	Cys	Glu	Cys	Ile	Glu	Gly	Phe	His	Cys	Leu	Gly	Pro
				85					90				95			
25	Gln	Cys	Thr	Arg	Cys	Glu	Lys	Asp	Cys	Arg	Pro	Gly	Gln	Glu	Leu	Thr
				100					105				110			
30	Lys	Gln	Gly	Cys	Lys	Thr	Cys	Ser	Leu	Gly	Thr	Phe	Asn	Asp	Gln	Asn
				115					120				125			
35	Gly	Thr	Gly	Val	Cys	Arg	Pro	Trp	Thr	Asn	Cys	Ser	Leu	Asp	Gly	Arg
				130					135				140			
40	Ser	Val	Leu	Lys	Thr	Gly	Thr	Thr	Glu	Lys	Asp	Val	Val	Cys	Gly	Pro
				145					150				155		160	
45	Pro	Val	Val	Ser	Phe	Ser	Pro	Ser	Thr	Thr	Ile	Ser	Val	Thr	Pro	Glu
															175	
50	Gly	Gly	Pro	Gly	Gly	His	Ser	Leu	Gln	Val	Leu	Thr	Leu	Phe	Leu	
									180				185		190	

35 (2) INFORMATION FOR SEQ ID NO:137:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 54 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

45

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTAC

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(2) INFORMATION FOR SEQ ID NO:138:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 380 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His
1 5 10 15

Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His
20 25 30

Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser
35 40 45

Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro
50 55 60

Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His
65 70 75 80

Asn Arg Val Cys Glu Cys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
85 90 95

Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala
100 105 110

Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe
115 120 125

Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn
130 135 140

Cys Ser Thr Phe Gly Leu Leu Ile Gln Lys Gly Asn Ala Thr His
145 150 155 160

Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile
165 170 175

Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr
180 185 190

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	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val	Asp	Ser	Leu	Pro	Gly
	195						200					205				
5	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	Lys	Arg	Arg	His	Ser
	210					215					220					
10	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu	Trp	Lys	His	Gln	Asn
	225					230				235					240	
15	Arg	Asp	Gln	Glu	Met	Val	Lys	Lys	Ile	Ile	Gln	Asp	Ile	Asp	Leu	Cys
	245					250				255						
20	Glu	Ser	Ser	Val	Gln	Arg	His	Leu	Gly	His	Ser	Asn	Leu	Thr	Thr	Glu
	260					265				270						
25	Gln	Leu	Leu	Ala	Leu	Met	Glu	Ser	Leu	Pro	Gly	Lys	Lys	Ile	Ser	Pro
	275					280				285						
30	Glu	Glu	Ile	Glu	Arg	Thr	Arg	Lys	Thr	Cys	Lys	Ser	Ser	Glu	Gln	Leu
	290					295				300						
35	Leu	Lys	Leu	Leu	Ser	Leu	Trp	Arg	Ile	Lys	Asn	Gly	Asp	Gln	Asp	Thr
	305					310				315					320	
40	Leu	Lys	Gly	Leu	Met	Tyr	Ala	Leu	Lys	His	Leu	Lys	Thr	Ser	His	Phe
	325					330				335						
45	Pro	Lys	Thr	Val	Thr	His	Ser	Leu	Arg	Lys	Thr	Met	Arg	Phe	Leu	His
	340					345				350						
50	Ser	Phe	Thr	Met	Tyr	Arg	Leu	Tyr	Gln	Lys	Leu	Phe	Leu	Glu	Met	Ile
	355					360				365						
55	Gly	Asn	Gln	Val	Gln	Ser	Val	Lys	Ile	Ser	Cys	Leu				
	370					375				380						

(2) INFORMATION FOR SEQ ID NO:139:

40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 380 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Glu	Glu	Thr	Ser	His
1						5					10				15	
5																
	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro	Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His
						20				25				30		
10																
	Cys	Thr	Ala	Lys	Trp	Lys	Thr	Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr
							35			40				45		
15																
	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro
						50			55			60				
20																
	Val	Cys	Lys	Glu	Leu	Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His
						65			70			75			80	
25																
	Asn	Arg	Val	Cys	Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe
							85			90				95		
30																
	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala
							100			105				110		
35																
	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe
							115			120				125		
40																
	Phe	Ser	Asn	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Arg	Lys	His	Thr	Asn
							130			135				140		
45																
	Cys	Ser	Val	Phe	Gly	Leu	Leu	Leu	Thr	Gln	Lys	Gly	Asn	Ala	Thr	His
							145			150				155		160
50																
	Asp	Asn	Ile	Cys	Ser	Gly	Asn	Ser	Glu	Ser	Thr	Gln	Lys	Cys	Gly	Ile
							165			170				175		
55																
	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg	Phe	Ala	Val	Pro	Thr
							180			185				190		
60																
	Lys	Phe	Thr	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val	Asp	Asn	Leu	Pro	Gly
							195			200				205		
65																
	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	Lys	Arg	Gln	His	Ser
							210			215				220		
70																
	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu	Trp	Lys	His	Gln	Asn
							225			230				235		240
75																
	Lys	Ala	Gln	Asp	Ile	Val	Lys	Lys	Ile	Ile	Gln	Asp	Ile	Asp	Leu	Cys
							245			250				255		

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Glu Asn Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu
260 265 270

5 Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala
275 280 285

Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile
290 295 300

10 Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr
305 310 315 320

Leu Lys Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe
325 330 335

15 Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His
340 345 350

Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile
20 355 360 365

Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu
370 375 380

25 (2) INFORMATION FOR SEQ ID NO:140:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

40 TGGACCACCC AGAACGTACCT TCATTATGAC 30

(2) INFORMATION FOR SEQ ID NO:141:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

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5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

GTCATAATGA AGGTACTTCT GGGTGGTCCA

30

10 (2) INFORMATION FOR SEQ ID NO:142:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

25 GGACCACCCA GCTTCATTAT GACGAAGAAA C

31

(2) INFORMATION FOR SEQ ID NO:143:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

GTTTCTTCGT CATAATGAAG CTGGGTGGTC C

31

45 (2) INFORMATION FOR SEQ ID NO:144:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50

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(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

GTGGACCACC CAGGACGAAG AAACCTCTC

29

10 (2) INFORMATION FOR SEQ ID NO:145:

15

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:

GAGAGGTTTC TTCGTCTGG GTGGTCCAC

29

25

(2) INFORMATION FOR SEQ ID NO:146:

30

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:

40

CGTTTCCTCC AAAGTTCTT CATTATGAC

29

(2) INFORMATION FOR SEQ ID NO:147:

45

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:

GTCATAATGA AGGAACCTTG GAGGAAACG

29

(2) INFORMATION FOR SEQ ID NO:148:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:

GGAAACGTTT CCTGCAAAGT ACCTTCATTA TG

32

25 (2) INFORMATION FOR SEQ ID NO:149:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:

40 CATAATGAAG GTACTTGCA GGAAACGTTT CC

32

(2) INFORMATION FOR SEQ ID NO:150:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 50 (ii) MOLECULE TYPE: cDNA

- 222 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:

5 CACGGAAAAG TCGGGAATAG ATGTCAC

27

(2) INFORMATION FOR SEQ ID NO:151:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:

GTGACATCTA TTCCCGACTT TTGCGTG

27

(2) INFORMATION FOR SEQ ID NO:152:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:

CACCCTGTCTG GAAGAGGCCT TCTTC

25

(2) INFORMATION FOR SEQ ID NO:153:

- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

- 223 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:153:

GAAGAAGGCC TCTTCCGACA GGGTG

25

(2) INFORMATION FOR SEQ ID NO:154:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:

TGACCTCTCG GAAAGCAGCG TGCA

24

25 (2) INFORMATION FOR SEQ ID NO:155:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:

40 TGCACGCTGC TTTCCGAGAG GTCA

24

(2) INFORMATION FOR SEQ ID NO:156:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

- 224 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:

5

CCTCGAAATC GAGCGAGCAG CTCC

24

(2) INFORMATION FOR SEQ ID NO:157:

10

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:

CGATTCGAG GTCTTCTCG TTCTC

25

25

(2) INFORMATION FOR SEQ ID NO:158:

30

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:

40

CCGTGAAAAT AAGCTCGTTA TAACTAGGAA TGG

33

(2) INFORMATION FOR SEQ ID NO:159:

45

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

- 225 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:

5 CCATTCTAG TTATAACGAG CTTATTTCA CGG

33

(2) INFORMATION FOR SEQ ID NO:160:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:160:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:161:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:161:

CCTCTCTCGA GTCAGGTGAC ATCTATTCCA CACTTTGCG TGGC

44

40 (2) INFORMATION FOR SEQ ID NO:162:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

- 226 -

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:162:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

10 (2) INFORMATION FOR SEQ ID NO:163:

10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:163:

CCTCTCTCGA GTCAAGGAAC AGCAAACCTG AAGAAGGC

38

25

(2) INFORMATION FOR SEQ ID NO:164:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:164:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:165:

45

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

- 227 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:165:

5 CCTCTCTCGA GTCACTCTGT GGTGAGGTTC GAGTGGCC

38

(2) INFORMATION FOR SEQ ID NO:166:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:166:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:167:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:167:

CCTCTCTCGA GTCAGGATGT TTTCAAGTGC TTGAGGGC

38

40 (2) INFORMATION FOR SEQ ID NO:168:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

50

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:168:

Met Lys His His His His His Ala Ser Val Asn Ala Leu Glu
1 5 10 15

(Ref ID: 1249 Pub 415)

FORM 13-27

13-205

Applicant's or agent's file
reference number

A-378-CIP2

International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)..

A. The indications made below relate to the microorganism referred to in the description on page 15, 16, 45, 50, 64, 67, 100 line 100 many to list.

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

American Type Culture Collection (ATCC)

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, MD 20852

Date of deposit

12/27/95 and 7/24/96

Accession Number

69969, 69970, 69971, 98112 and 98113

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")

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 This sheet was received with the international application

Authorized officer

Elvira Rivera
Patent Specialist
IAPC - PCT Coordinator
(703) 355-3678

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 This sheet was received by the International Bureau on:

Authorized officer

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WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding a
5 polypeptide comprising at least one of the biological
activities of OPG wherein the nucleic acid is selected
from the group consisting of:

- a) the nucleic acids shown in Figures 2B-
2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D
10 (SEQ ID NO:124) or complementary strands thereof;
- b) nucleic acids which hybridize under
stringent conditions with the polypeptide-encoding
regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B
(SEQ ID NO:122) and 9C-9D (SEQ ID NO:124);
- c) nucleic acids which hybridize under
stringent conditions with nucleotides 148 through 337
inclusive as shown in Figure 1A; and
- d) nucleic acid which are degenerate to
the nucleic acids of (a), (b) and (c).

20

2. The nucleic acid of Claim 1 which is cDNA,
genomic DNA, synthetic DNA or RNA.

25 3. A polypeptide encoded by the nucleic acid
of Claim 1.

4. The nucleic acid of Claim 1 including one
or more codons preferred for Escherichia coli
expression.

30

5. The nucleic acid of Claim 1 having a
detectable label attached thereto.

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6. The nucleic acid of Claim 1 comprising the polypeptide-encoding region of Figure 2B-2C (SEQ ID NO:120), Figure 9A-9B (SEQ ID NO:122) or Figure 9C-9D (SEQ ID NO:124).

5

7. The nucleic acid of Claim 6 having the sequence as shown in Figure 9C-D (SEQ ID NO:124) from nucleotides 158-1297.

10

8. An expression vector comprising the nucleic acid of Claim 1.

15

9. The expression vector of Claim 8 wherein the nucleic acid comprises the polypeptide - encoding region as shown in Figure 9C-9D (SEQ ID NO:124).

10. A host cell transformed or transfected with the expression vector of Claim 8.

20

11. The host cell of Claim 10 which is a eucaryotic cell.

25

12. The host cell of Claim 11 which is selected from the group consisting of CHO, COS, 293, 3T3, CV-1 and BHK cells.

13. The host cell of Claim 10 which is a procaryotic cell.

30

14. The host cell of Claim 13 which is Escherichia coli.

15. A transgenic mammal comprising the expression vector of Claim 8.

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16. The transgenic mammal of Claim 15 which is a rodent.

5 17. The transgenic mammal of Claim 16 which is a mouse.

18. A process for the production of OPG comprising:

10 growing under suitable nutrient conditions host cells transformed or transfected with the nucleic acid of Claim 1; and

isолating the polypeptide products of the expression of the nucleic acids.

15

19. A purified and isolated polypeptide comprising OPG.

20 20. The polypeptide of Claim 19 which is mammalian OPG.

21. The polypeptide of Claim 20 which is human OPG.

25 22. The polypeptide of Claim 19 which is substantially free of other human proteins.

30 23. The polypeptide of Claim 21 having the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123), or Figure 9C-9D (SEQ ID NO:125) or a derivative thereof.

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24. The polypeptide of Claim 23 having the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-401 inclusive.

5 25. The polypeptide of Claim 23 having the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 32-401 inclusive.

10 26. The polypeptide of Claim 19 which is characterized by being a product of expression of an exogenous DNA sequence.

15 27. The polypeptide of Claim 26 wherein the DNA is cDNA, genomic DNA or synthetic DNA.

28. The polypeptide of Claim 19 which has been modified with a water-soluble polymer.

20 29. The polypeptide of Claim 28 wherein the water soluble polymer is polyethylene glycol.

30 30. A polypeptide comprising:
an amino acid sequence of at least about 164
amino acids comprising four cysteine-rich domains
25 characteristic of the cysteine rich domains of tumor
necrosis factor receptor extracellular regions; and
an activity of increasing bone density.

30 31. A polypeptide comprising the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121),
Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID NO:125) having an amino terminus at residue 22, and
wherein from 1 to 216 amino acids are deleted from the carboxy terminus.

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32. The polypeptide of Claim 31 comprising the amino acid sequence from residues 22-185, 22-189, 22-194, or 22-201 inclusive.

5

33. The polypeptide of Claim 32 further comprising an Fc region of human IgG1 extending from the carboxy terminus.

10

34. A polypeptide comprising the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID NO:125) having an amino terminus at residue 22, wherein from 1 to 10 amino acids are deleted from the amino terminus and, optionally, from 1 to 216 amino acids are deleted from the carboxy terminus.

15

35. The polypeptide of Claim 34 comprising the amino acid sequence from residues 27-185, 27-189, 27-194, 27-401, or 32-401 inclusive.

20

36. The polypeptide of Claim 35 further comprising an Fc region of human IgG1 extending from the carboxy terminus.

25

37. A polypeptide selected from the group consisting of:

huOPG [22-201]-Fc

huOPG [22-401]-Fc

30

huOPG [22-180]-Fc

huOPG met [22-401]-Fc

huOPG Fc-met [22-401]

huOPG met [22-185]

huOPG met [22-189]

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huOPG met [22-194]
huOPG met [27-185]
huOPG met [27-189]
huOPG met [27-194]
5 huOPG met [32-401]
huOPG met-lys[22-401]
huOPG met [22-401]
huOPG met [22-401]-Fc (P25A)
huOPG met [22-401] (P25A)
10 huOPG met [22-401] (P26A)
huOPG met [22-401] (P26D)
huOPG met [22-194] (P25A)
huOPG met [22-194] (P26A)
huOPG met met-(lys)3 [22-401]
15 huOPG met met-arg-gly-ser-(his)6 [22-401]

38. A nucleic acid encoding the polypeptide
of Claim 37.

20 39. An antibody or fragment thereof which
specifically binds to OPG.

40. The antibody of Claim 39 which is a
monoclonal antibody.

25 41. A method for detecting the presence of
OPG in a biological sample comprising:
incubating the sample with the antibody of
Claim 39 under conditions that allow binding of the
30 antibody to OPG; and
detecting the bound antibody.

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42. A method to assess the ability of a candidate substance to bind to OPG comprising:
incubating OPG with the candidate substance
5 under conditions that allow binding; and
measuring the bound substance.

43. A method of regulating the levels of OPG in an animal comprising modifying the animal with a
10 nucleic acid encoding OPG.

44. The method of Claim 43 wherein the nucleic acid promotes an increase in the tissue level of OPG.
15

45. The method of Claim 44 wherein the animal is a human.

46. A pharmaceutical composition comprising a
20 therapeutically effective amount of OPG in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.

47. The composition of Claim 46 wherein the
25 OPG is human OPG.

48. The composition of Claim 47 wherein the OPG has the amino acid sequence as shown in Figure 9B.

30 49. A method of treating a bone disorder comprising administering a therapeutically effective amount of the polypeptide of Claim 19.

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50. The method of Claim 49 wherein the polypeptide is human OPG.

51. The method of Claim 49 wherein the bone disorder is excessive bone loss.

52. The method of Claim 51 wherein the bone disorder is selected from the group consisting of osteoporosis, Paget's disease of bone, hypercalcemia, 10 hyperparathyroidism, steroid-induced osteopenia, bone loss due to rheumatoid arthritis, bone loss due to osteomyelitis, osteolytic metastasis, and periodontal bone loss.

15 53. The method of Claim 49 further comprising administering a therapeutically effective amount of a substances selected from the group consisting of bone morphogenic proteins BMP-1 through BMP-12, TGF- β family members, IL-1 inhibitors, TNF α inhibitors, parathyroid 20 hormone and analogs thereof, parathyroid hormone related protein and analogs thereof, E series prostaglandins, bisphosphonates, and bone-enhancing minerals.

25 54. An osteoprotegerin multimer consisting of osteoprotegerin monomers.

55. The multimer of Claim 54 which is a dimer.

30 56. The multimer of Claim 54 formed by interchain disulfide bonds.

57. The multimer of Claim 54 formed by association Fc regions derived from human IgG1.

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58. The multimer of Claim 54 which is essentially free of osteoprotegerin monomers and inactive multimers.

5

59. The multimer of Claim 54 wherein the monomers comprise the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-401, or a derivative thereof.

10

60. The multimer of Claim 54 wherein the monomers comprise the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-194.

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FIG. 1A

FRI-1	148	178	208	238	268	298
	ALLVFLDIIIEWTTQETFPKKYLHYDPETGRQLLCDKCAPGTYLKQHCTVRRKTLCVCPD					
UMAN	HALPAQVAFTPYAPEGSTCRLREYYDQTAQMCCSKCSPGQHAKVFCTKTSDTVCDSCED	30	40	50	60	70

STYTQLWNWPECLSGSRCSSDQVETQACTREQNRICTCRPGWYCALSKQEGRCLCPL
90 100 110 120 130 140
SW : TNR2 _HUMAN

FIG. 1B

FRI-1	69	YLHYDPEGRQLLCCDKCAPGTYLKQHC . TVRRKTLGV . PCPDY . SYTDSW
TNFR profile	6	YHYDQNGRMCEECHMCQPGHFLVKHCKQPKRDTVCHKPCEPGVTRYTDDW
FRI-1	116	H
TNFR profile	56	H

$$Z \text{ Score} = 8.29$$

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FIG. IC

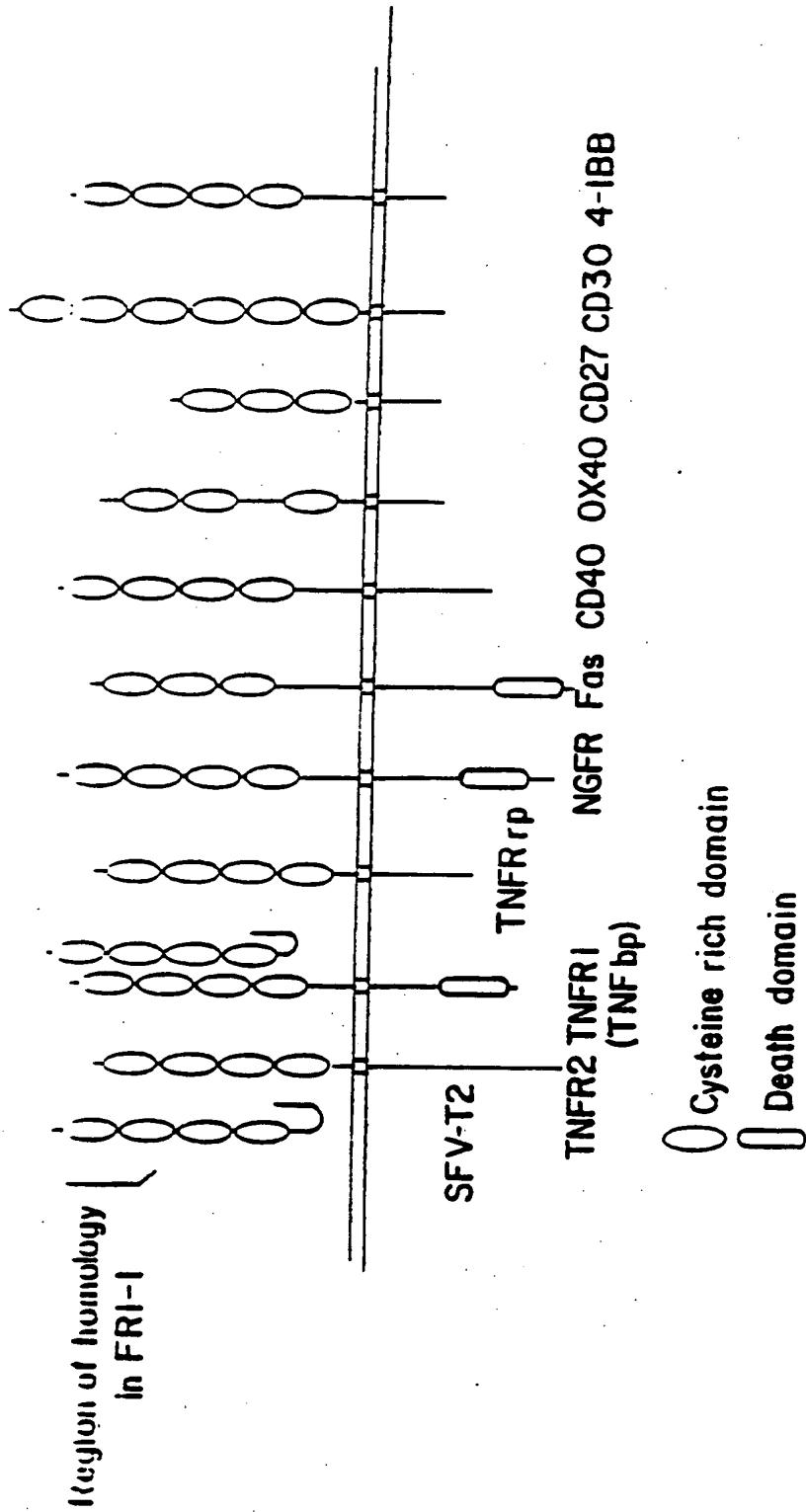


FIG.2A

AUG

TAG

58

FIG. 2B

10	30	50
ATCAAAGGCAGGGCATACTTCCGTGCCCCAGACCTTATATAAAACGTCATTTGCCCTG		
70	90	110
GGCACCGAGAGAACGACCTAGCACTGGCCCAGCGGCTGCCCTGAGGTTCCAGAGGACC		
130	150	170
ACAATGAACAAAGTGGCTGTGCTGTGCACTCCTGGTGTGACATCATTGAATGGACA		
M N K W L C C A L L V F L D I E W T		
190	210	230
ACCCAGGAAACCTTCTCCAAAATACTTGCAATTATGACCCAGAAACCGGACGTCAGCTC		
T O E T F P P K Y L H Y D P E T G R Q L		
250	270	290
TTGTGTGACAAATGTGCTCTGGCACCTACCTAAGCACTGCACAGTCAGGAGGAAG		
L C D K C A P G T Y L K Q H C T V R R R K		
310	330	350
ACACTGTGTGTCCTGCCCTGACTACTCTTATACAGACAGCTGGCACACGAGTGATGAA		
T L C V P C P D Y S Y T D S W H T S D E		
370	390	410
TGCGTGTACTGCAGCCCCGTGTGCAAGGAACGTGAGACCGTGAAACAGGAGTGCAACCGC		
C V Y C S P V C K E L Q T V K Q E C N R		
430	450	470
ACCCACAACCGAGTGTGCAATGTGAGGAAGGGCGCTACCTGGAGCTCGAATTCTGCTTG		
T H N R V C E C E E G R Y L E L E F C L		
490	510	530
AAGCACCGGAGCTGTCCCCCAGGCTTGGGTGTGCTGCAGGCTGGACCCAGAGCGAAAC		
K H R S C P P G L G V L Q A G T P E R N		
550	570	590
ACGGTTTGCAAAAGATGTCCGGATGGGTCTTCTCAGGTGAGACGTATCGAAAGCACCC		
T V C K R C P D G F F S G E T S S K A P		
610	630	650
TGTAGGAAACACACCAACTGCAGCTCACCTGGCTCCTGCTAATTCAAGAAAGGAAATGCA		
C R K H T N C S S L G L L I Q K G N A		
670	690	710
ACACATGACAATGTATGTTCCGGAAACAGAGAACGAGCAACTCAAAATTGTGGAATAGATGTC		
T H D N V C S G N R E A T Q N C G I D V		
730	750	770
ACCCTGTGCGAAGAGGCATTCTCAGGTTGCTGTGCTACCAAGATTATACCGAATTGG		
T L C E E A F F R F A V P T K I I P N W		
790	810	830
CTGAGTGTCTGGGACAGTTGCCCTGGACCAAAAGTGAATGCAGAGAGTGTAGAGAGG		
L S V L V D S L P G T K V N A E S V E R		
850	870	890
ATAAAACGGAGACACAGCTCGCAAGAGCAAACCTTCCAGCTACTTAAGCTGTGGAAGCAT		
I K R R H S S Q E Q T F Q L L K L W K H		
910	930	950
CAAAACAGAGACCAGGAAATGGTGAAGAACGATCATCCAAGACATTGACCTCTGTGAAAGC		
Q N R D Q E M V K K I I Q D I D L C E S		
970	990	1010
AGTGTGCAACGGCATATCGGCCACCGAACCTCACCAAGAGCAGCTCCGCATTTGATG		
S V Q R H I G H A N L T T E Q L R I L M		

FIG.2C

1030	1050	1070
GAGAGCTGCCTCGGAAGAAGATCAGCCCAGACGAGATTGAGAGAACGAGAAAGACCTGC		
E S L P G K K I S P D E I E R T R K T C		
1090	1110	1130
AAACCCAGCGAGCAGCTCCTGAAGCTACTGAGCTTGTGGAGGATCAAAAATGGAGACCAA		
K P S E Q L L K L L S L W R I K N G D Q		
1150	1170	1190
GACACCTTGAAGGGCCTGATGTACGCCACTCAAGCACTTGAAAGCATAACCACCTTCCAAA		
D T L K G L M Y A L K H L K A Y H F P K		
1210	1230	1250
ACCGTCACCCACAGTCTGAGGAAGACCATCAGGTTCTGCACAGCTTACCATGTACCGA		
T V T H S L R K T I R F L H S F T M Y R		
1270	1290	1310
TTGTATCAGAAACTCTTCTAGAAATGATAGGAAATCAGGTTCAATCAGTGAAGATAAGC		
L Y Q K L F L E M I G N Q V Q S V K I S		
1330	1350	1370
TGCTTATAGTTAGGAATGGTCACTGGGCTTTCTTCAGGATGGGCCAACACTGATGGAG		
C L		
1390	1410	1430
CAGATGGCTGCTTCTCCGGCTTTGAAATGGCAGTTGATTCTCATCAGTTGGTGG		
1450	1470	1490
GAATGAAGATCCTCCAGCCAACACACACACTGGGAGTCTGAGTCAGGAGAGTGAGGCA		
1510	1530	1550
GGCTATTTGATAATTGTGCAAAGCTGCCAGGTGTACACCTAGAAAGTCAGCACCTGAG		
1570	1590	1610
AAAGAGGATATTTTATAACCTCAAACATAGGCCCTTCCTCTCCTTATGGATGAG		
1630	1650	1670
TACTCAGAAGGCTTCTACTATCTCTGTGTCATCCCTAGATGAAGGCCTCTTTATTTAT		
1690	1710	1730
TTTTTTATTCTTTTCGGAGCTGGGACCGAACCCAGGGCTTGCCTTGCAGGCAA		
1750	1770	1790
GTGCTCTACCACTGAGCTAAATCTCCAACCCCTGAAGGCCTCTTCTGCCTCTGAT		
1810	1830	1850
AGTCTATGACATTCTTTCTACAATTGCTATCAGGTGCACGAGCCTATCCCATTGAT		
1870	1890	1910
AGGTTCTAGGCAAGTTGACCGTTAGCTATTTCCCTCTGAAGATTGATTGAGTTGC		
1930	1950	1970
AGACTTGGCTAGACAAGCAGGGTAGGTTAGTTATTTAACAGACTGCCACCAGG		
1990	2010	2030
AGTCCAGTCTTCTGTTCTGTAGTTGACTTAAGCTGACTCCAAGTACATTAGTA		
2050	2070	2090
TGAATAATCAACAAATTATTCTCTATCAACATTGGCTAGCTTGTTCAGGGC		
2110	2130	2150
ACTAAAAGAAACTACTATATGGAGAAAGAATTGATATTGCCCAACGTTCAACAAACCA		
2170	2190	2210
ATAGTTTATCCAGCTGTATGCCCTGGTCAGTGTACTGACTATGCCCTTATTAC		
2230	2250	2270
TGCATGCAGTAATTCAACTGGAAATAGTAATAATAATAGAAATAATCTAGACTCC		
2290	2310	2330
ATTGGATCTCTCTGAATATGGAAATCTAACCTAACAGCTTGTGAGATTTCAGTTGTGT		
2350	2370	2390
TAAAGGCTTTATTAAAAAGCTGATGCTCTGTAAAAGTTACTAATATCTGTAAGA		
2410	2430	
CTATTACAGTATTGCTATTATCCATCCAG		

FIG. 2D

fas.frg	H F -	- - S S K C R R -	- - -	103
tnf1.frg	H L R H C L S C R K E M G Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F Q C F	- - -	- - -	144
sfv-t2.frg	H - - A P A C V S C R G -	- - -	- - -	-
tnfir2.frg	H - - V P E C L S C G S -	- - -	- - -	-
cd40.frg	R E I R C H Q H R H C E P -	- - -	- - -	98
osteo.frg	- - T S D E C V Y C S P -	- - -	- - -	85
ngfr.frg	A T E P C K P C T E C -	- - -	- - -	65
ox40.frg	- Y D T C K Q C T O C N H -	- - -	- - -	89
4lbb.frg	- I G G Q P N C N T C R -	- - -	- - -	84
				65

FIG. 2E

fas.frg	- C R I L C D E G H G L E V E I N C T R T O N T K C R C K P N F F C N S T V C E H C D P C T K C E H G	152	
tnfr1.frg	N C S I L C L N G - - - - - P C T G H I S S Q P C D R I T H D R V C N C S T G N Y C L L K G O N G C R L C A P L R K C	191	
sfv-t2.frg	- - - - - R C S S D O V E T O A C T R E Q N R I C T V C T E S D T V C T E C E C A Y G Y Y - - - - - Q D E T T G R C E A C R V C	129	
tnfr2.frg	- - - - - N Q G L R V K K E G T A E S D T H N R V C R C A Y G Y Y - - - - - R P G T Q P R Q D S S H - - - E K D C	143	
cd40.frg	osteo.frg	- - - - - V C K E L O T V K O E C N R T H D A V C R C A Y G Y Y - - - - - R S G S E L - - - V C A G Y E R F K K F C S S T H N A E C Z C I E G E H C L G P Q C T R C - - -	125
ngfr.frg	- - - - - V G L O S H - - - - - Q A C K P W T N C S T H N A E C Z C I E G E H C L G P Q C T R C - - -	124	
ox40.frg	- - - - - V G L O S H - - - - - Q A C K P W T N C S T H N A E C Z C I E G E H C L G P Q C T R C - - -	128	
4lbb.frg	- - - - - V G L O S H - - - - - Q A C K P W T N C S T H N A E C Z C I E G E H C L G P Q C T R C - - -	116	
fas.frg	- - - - - E G S R S N L - - - D S G T T V L L P L Y I F G T S F N Y T S V G F N L Y - - - G W L C L L P I P L I	105	
tnfr1.frg	- - - - - T K C K E - - - - - D S S L S P T E R C G T S T D I C R P H O I C N V A I P G N - - - - - C L L S I L P I P L I	187	
sfv-t2.frg	P A G Y G V S - G H T R A G D T L C E K C P P H T Y S N T T S S N O S S S G E T S S K A P C R K H T N C C S S L G L L I	230	
tnfr2.frg	R P G F G V M E M A T E T T D T V C K R C P D G F F S G E T S S K A P C R K H T N C C S S L G L L I	178	
cd40.frg	I P G F G V M E M A T E T T D T V C K R C P D G F F S G E T S S K A P C R K H T N C C S S L G L L I	193	
osteo.frg	P P G L G V L Q A G I T P E R N T V C K R C P D G F F S G E T S S K A P C R K H T N C C S S L G L L I	175	
ngfr.frg	P P G L G V L Q A G I T P E R N T V C K R C P D G F F S G E T S S K A P C R K H T N C C S S L G L L I	174	
ox40.frg	R P G Q E L T K Q G - - - - - K L G V D C V P C P P G H F S P G S N - - - Q A C K P W T N C S T H N A E C Z C I E G E H C L G P Q C T R C - - -	178	
4lbb.frg	R P G Q E L T K Q G - - - - - K L G V D C V P C P P G H F S P G S N - - - Q A C K P W T N C S T H N A E C Z C I E G E H C L G P Q C T R C - - -	152	
fas.frg	V W V - - - - - K R K E V Q K T C R K H R K E N Q G S H E S P T L N P E - - - - - G H N E V I K T K E E F T S P V T L N Y T - - - - - C E E A F F R	147	
tnfr1.frg	G L M Y R Y Q R K S K L Y S I V C G K S T P E K E G E L E G T T T K P L A P N P S F S P T P G F T - - - - - D A V C T S P T R S H A P G A V H I L P Q P V S T R S Q H T - - - - - T S Q T N V I C G N R E A T Q N C G I D V T L C E E A F F R	219	
sfv-t2.frg	P V N - - - - - E T S C T T I A - - - - - D A V C T S P T R S H A P G A V H I L P Q P V S T R S Q H T - - - - - N A T H D N V C S G N R E A T Q N C G I D V T L C E E A F F R	280	
tnfr2.frg	A S R - - - - - D A V C T S P T R S H A P G A V H I L P Q P V S T R S Q H T - - - - - P P E G S D S T A P S T Q E P E A P P E Q D L L I - - - - - W I T R S T I T F R P T V P S T T V W P R T S Q T E P S T T V Q V L Q V L	207	
cd40.frg	O K G - - - - - T S Q T N V I C G N R E A T Q N C G I D V T L C E E A F F R	227	
osteo.frg	Q K G - - - - - N A T H D N V C S G N R E A T Q N C G I D V T L C E E A F F R	197	
ngfr.frg	E C T R W A D A E C E E I P G R - - - - - W I T R S T I T F R P T V P S T T V Q V L Q V L	208	
ox40.frg	H P A S N S V C E D R S L L A T L L W E T Q R T V P S T T V Q V L Q V L	224	
4lbb.frg	K T G T T E K D V V C G P P V V S F S P S T T V Q V L Q V L	202	

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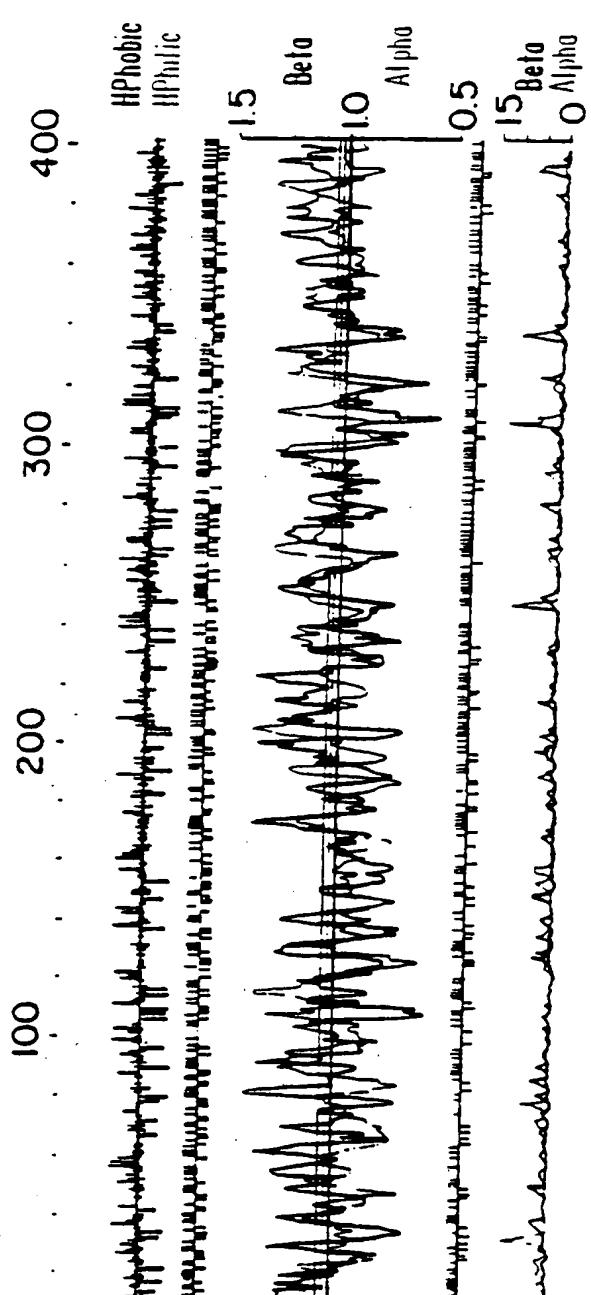


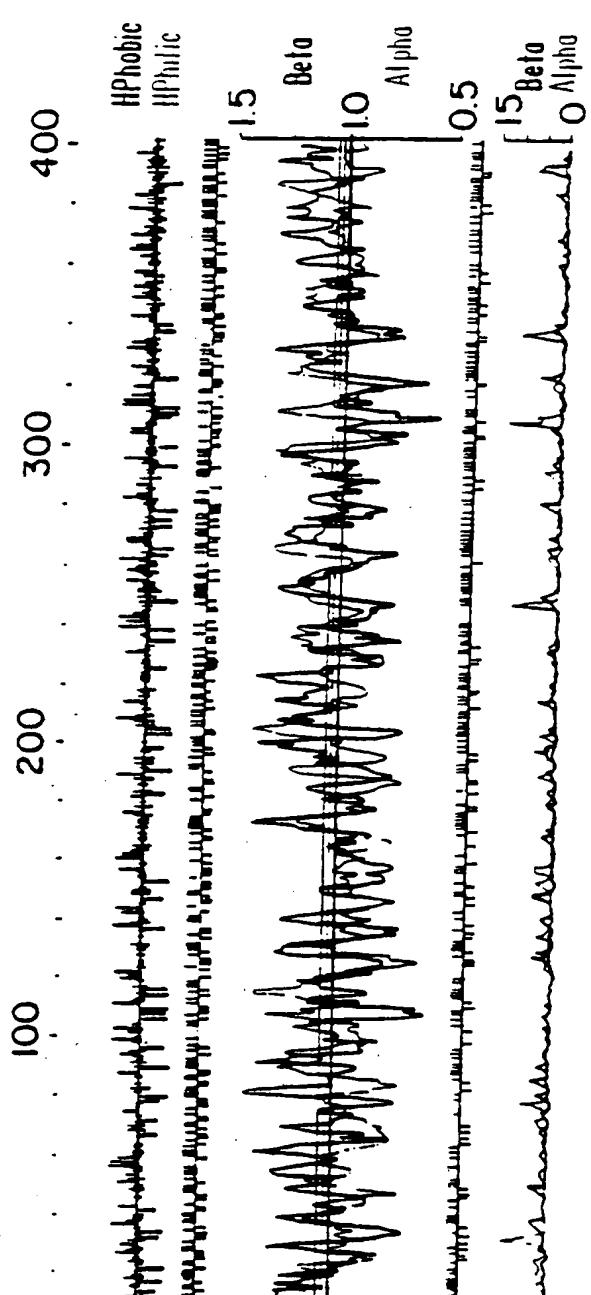
FIG. 3A

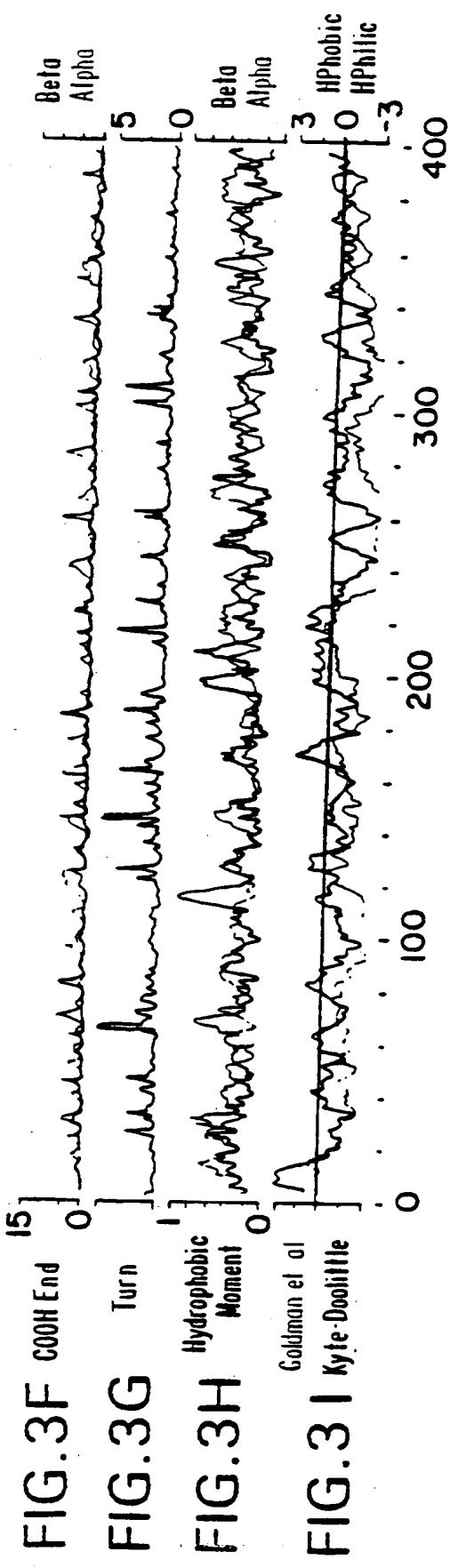
FIG. 3B

FIG. 3C

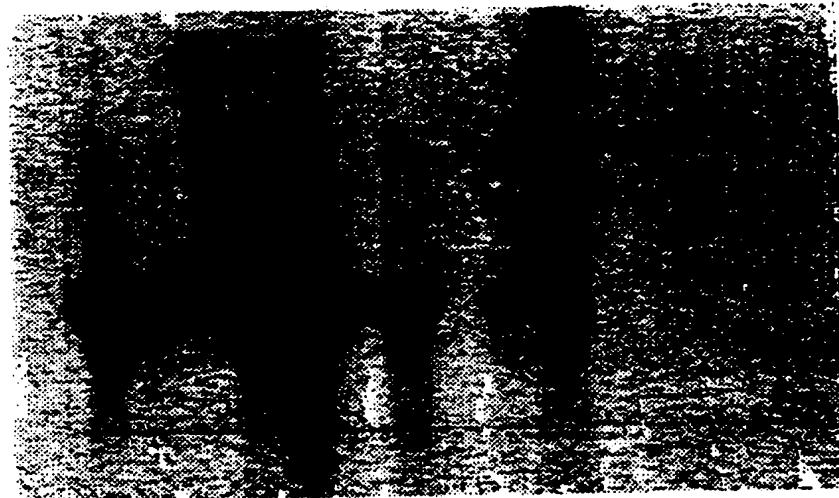
FIG. 3D

FIG. 3E





1 0 / 4 6

FIG.52 11 16 17 22 28 33 38 45 Kb**Transgenic Founders****Controls**

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FIG.4A

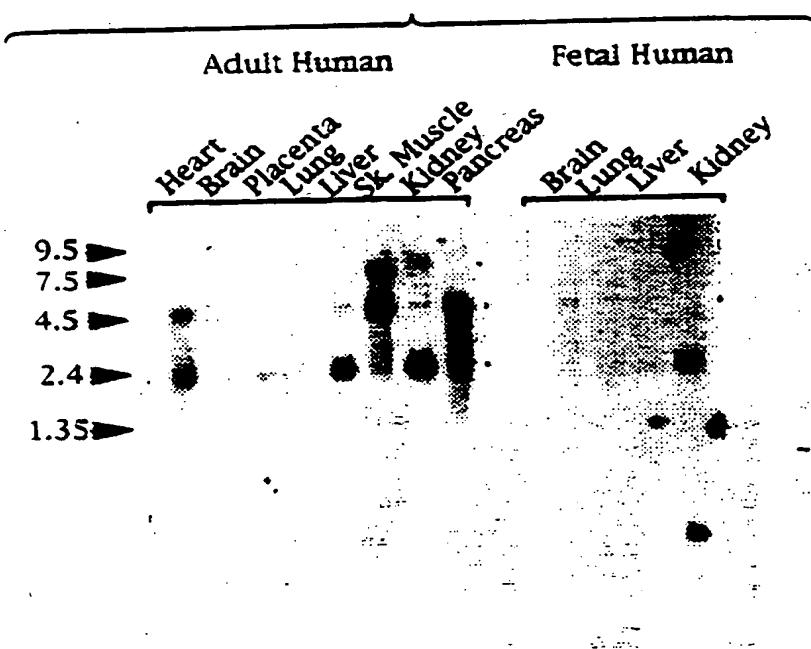


FIG.4B

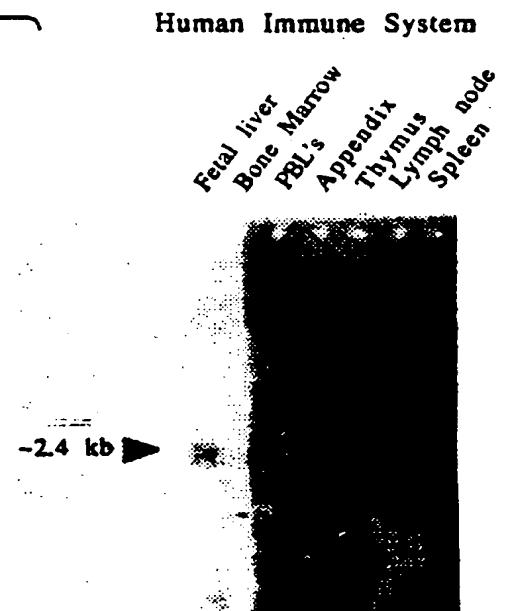


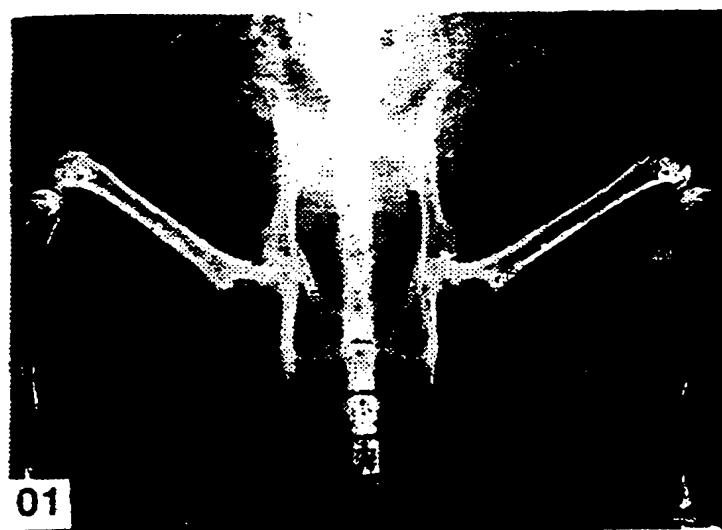
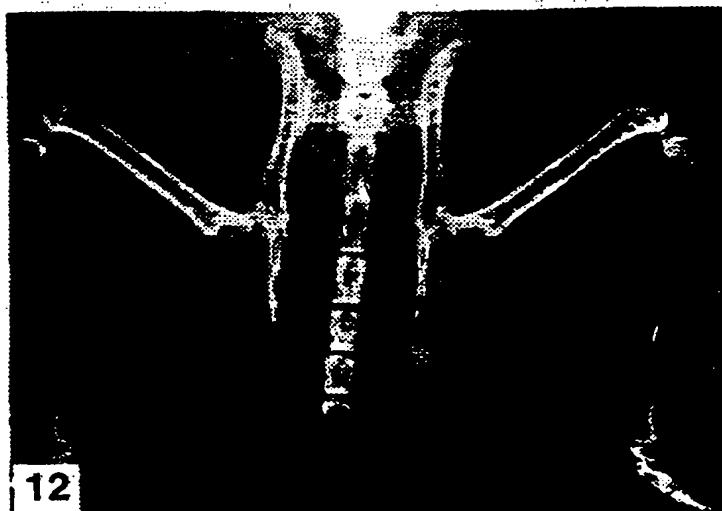
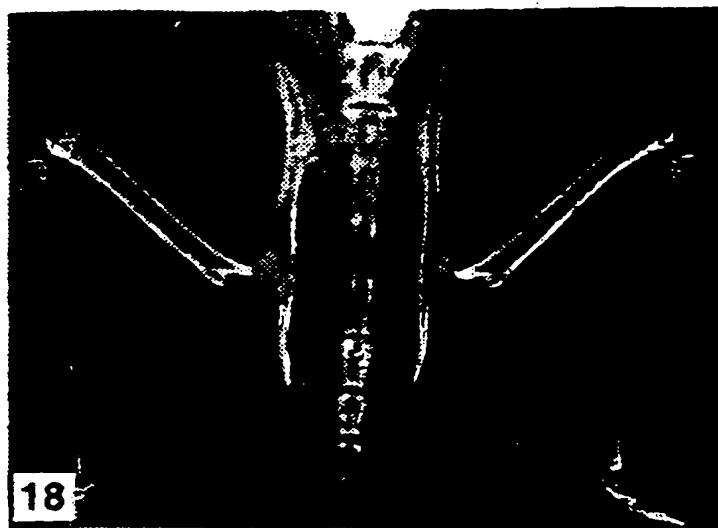
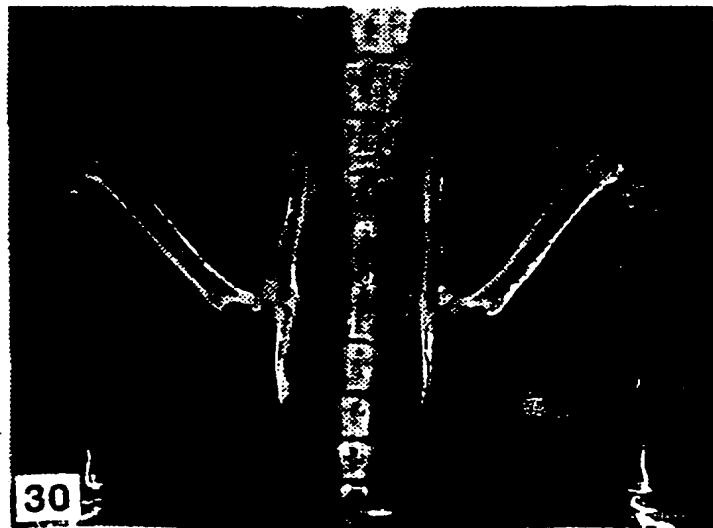
FIG.6A**FIG.6B****FIG.6C**

FIG.6D**FIG.6E****FIG.6F**

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FIG.6G



FIG.6H

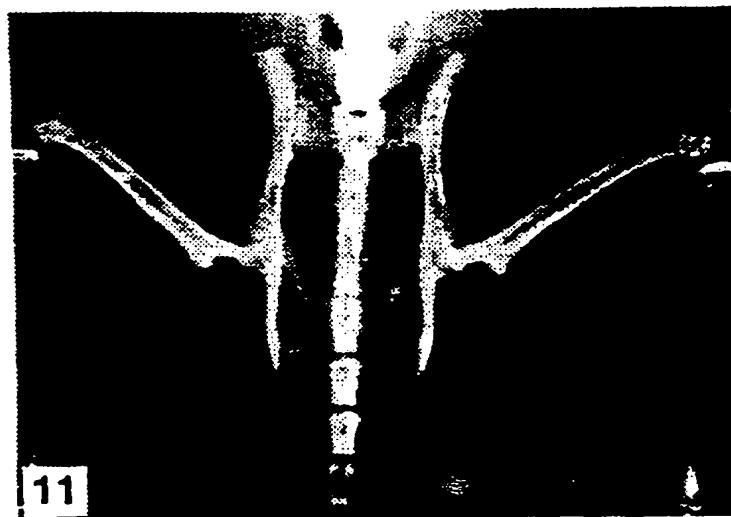


FIG. 9A

10 30 50
 CCTTATATAAACGTGATGGCTGGCTGCAGAGACGCACCTAGCACTGACCCAGCG
 70 90 110
 GCTGCCCTCCTGAGGTTCCGAGGACCACAATGAACAAAGTGGCTGTGCTGCCACTCCTG
 130 150 M N K W L C C A L L 170
 GTGCTCCTGGACATCATTAAGAATGGACAAACCCAGGAAACCCCTTCCTCAAAGTACTTGAT
 V L L D I I E W T T O E T L P P K Y L H
 190 210 230
 TATGACCCAGAAACTGGTCATCAGCTCTGTGAGACAAATGAGCTCCTGGCACCTACCTA
 Y D P E T G H Q L L C D K C A P G T Y L
 250 270 290
 AAACAGCACTGCACAGTGAGGAGGAAGACATTGTGTGTCCCTGCCCTGACCACTCTTAT
 K Q H C T V R R K T L C V P C P D H S Y
 310 330 350
 ACGGACAGCTGGCACACCAGTGATGAGTGTGTATTGCAGCCCAGTGTGCAAGGAAC TG
 T D S W H T S D E C V Y C S P V C K E L
 370 390 410
 CAGTCCTGTGAAGCAGGAGTGCAACCGCACCCACAACCGAGTGTGAGTGTGAGGG
 Q S V K Q E C N R T H N R V C E C E E G
 430 450 470
 CGTTACCTGGAGATCGAATTCTGCTTGAAGCACCGGAGCTGTCCCCGGCTCCGGCGTG
 R Y L E I E F C L K H R S C P P G S G V
 490 510 530
 GTGCAAGCTGAAACCCAGAGCGAAACACAGTTGCAAAAAATGTCCAGATGGGTTCTTC
 V Q A G T P E R N T V C K K C P D G F F
 550 570 590
 TCAGGTGAGACTTCATCGAAAGCACCCAGAGCGAAACACAGTTGCAAAAAATGTCCAGATGGGTTCTTC
 S G E T S S K A P C I K H T N C S T F G
 610 630 650
 CTCCTGCTAATTGAGAAAGGAAATGCAACACATGACAACGTGTGTTCCGGAAACAGAGAA
 L L L I Q K G N A T H D N V C S G N R E
 670 690 710
 GCCACGCCAAAAGTGTGGAATAGATGTCAACCTGTGAGAGGGCTTCTTCAGGTTGCT
 A T Q K C G I D V T L C E E A F F R F A
 730 750 770
 GTTCCTACCAAGATTACCAAATTGGCTGAGTGTGTTGGACAGTTGCCCTGGAC
 V P T K I I P N W L S V L V D S L P G T

FIG.9B

790	810	830
AAAGTGAATGCCGAGAGTAGAGAGGATAAAACGGAGACACAGCTCACAAAGAGCAAACC		
K V N A E S V E R I K R R H S S Q E Q T		
850	870	890
TTCCAGCTGCTGAAGCTGTGGAAACATCAAAACAGAGACCAGGAAATGGTGAAGAAGATC		
F Q L L K L W K H Q N R D Q E M V K K I		
910	930	950
ATCCAAGACATTGACCTCTGTGAAAGCAGCGTGCAGCGGCATCTCGGCCACTCGAACCTC		
I Q D I D L C E S S V Q R H L G H S N L		
970	990	1010
ACCACAGAGCAGCTCTTGCCTTGATGGAGAGCCTGCCTGGGAAGAAGATCAGCCCAGAA		
T T E Q L L A L M E S L P G K K I S P E		
1030	1050	1070
GAGATTGAGAGAACGAGAAAGACCTGCAAATCGAGCGAGCAGCTCTGAAGCTACTCAGT		
E I E R T R K T C K S S E Q L L K L L S		
1090	1110	1130
TTATGGAGGATCAAAAATGGTGAACCAAGACACCTTGAAGGGCCTGATGTATGCCCTCAAG		
I W R I K N G D Q D T L K G L M Y A L K		
1150	1170	1190
CACTTGAAAACATCCCACTTTCCTCAAAACTGTCAACCCACAGTCTGAGGAAGACCATGAGG		
H L K T S H F P K T V T H S L R K T M R		
1210	1230	1250
TTCCCTGCACAGCTTCACAATGTACAGACTGTATCAGAAGCTCTTTAGAAATGATAGGG		
F L H S F T M Y R L Y Q K L F L E M I G		
1270	1290	1310
AATCAGGTTCAATCCGTAAAATAAGCTGCTTATAACTAGGAATGGTCACTGGGCTGTT		
N Q V Q S V K I S C L		

CTTCA

FIG. 9C

10
 GTATATATAACGTGATGAGCGTACGGGTGCGGAGACGCACCGGAGCGCTCGCCCAGCCGC
 70
 CGYCTCCAAGCCCCCTGAGGTTCCGGGGACCACAATGAACAAAGTTGCTGTGCTGCCGCT
 130
 CGTGTTCTGGACATCTCCATTAAAGTGGACCACCCAGGAAACGTTCCCTCAAAGTACCT
 V F L D I S I K W T T O E T F P P K Y L
 190
 TCATTATGACGAAGAACCTCTCATCAGCTGTTGTGACAAATGTCCTCCCTGGTACCTA
 H Y D E E T S H Q L L C D K C P P G T Y
 250
 CCTAAACAAACACTGTACAGCAAAGTGGAAAGACCGTGTGCGCCCTTGCCCTGACCACTA
 L K Q H C T A K W K T V C A P C P D H Y
 310
 CTACACAGACAGCTGGCACACCAAGTGAACGAGTGTCTATACTGCAGCCCCGTGTGCAAGGA
 Y T D S W H T S D E C L Y C S P V C K E
 370
 GCTGCAGTACGTCAAGCAGGAGTGCACCGTGTGCGAATGCAAGGA
 L Q Y V K Q E C N R T H N R V C E C K E
 430
 AGGGCGCTACCTTGAGATAGAGTTCTGCTTGAAACATAGGAGCTGCCCTGGATTTGG
 G R Y L E I E F C L K H R S C P P G F G
 490
 AGTGGTGCAGCTGGAACCCCCAGAGCAGAAATACAGTTGCAAAAGATGTCAGATGGTT
 V V Q A G T P E R N T V C K R C P D G F
 550
 CTTCTCAAATGAGACGTACCTAAAGCACCCCTGTAGAAAACACACAAATTGCAAGTGTCTT
 F S N E T S S K A P C R K H T N C S V F
 610
 TGGTCTCCTGCTAACTCAGAAAGGAAATGCAACACACGACAACATATGTTCCGGAAACAG
 G L L L T Q K G N A T H D N I C S G N S
 670
 TGAATCAACTCAAAATGTGGAATAGATGTTACCCGTGTGAGGAGGCATTCTCAGGTT
 E S T Q K C G I D V T L C E E A F F R F
 730
 TGCTGTTCCCTACAAAGTTACGCCACTGGCTTAGTGTCTGGTAGACAATTGCCCTGG
 A V P T K F T P N W L S V L V D N L P G

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FIG.9D

790 CACCAAAGTAAACGCCAGAGAGTGTAGAGAGGATAAAACGGCAACACAGCTCACAAGAACAA T K V N A E S V E R I K R Q H S S Q E Q	810 850 GACTTTCCAGCTGCTGAAGTTATGGAAACATCAAAACAAAGACCAAGATATAGTCAGAA T F Q L L K L W K H Q N K D Q D I V K K	830 870 890 910 GATCATCCAAGATATTGACCTCTGTGAAAACAGCGTGCAGCGGCACATTGGACATGCTAA I I Q D I D L C E N S V Q R H I G H A N
930 950 970 CCTCACCTTCGAGCAGCTTCGTAGCTTGATGGAAAGCTTACCGGGAAAGAAAGTGGGAGC L T F E Q L R S L M E S L P G K K V G A	990 1010 1030 AGAAGACATTGAAAAAAACAATAAAGGCATGCACCCAGTGACCAGATCCTGAAGCTGCT E D I E K T I K A C K P S D Q I L K L L	1050 1070 1090 1110 1130 CAGTTTGTGGCGAATAAAAATGGCGACCAAGACACCTTGAGGGCTTAATGCACGC S L W R I K N G D Q D T L K G L M H A L
1150 1170 1190 AAAGCACTCAAAGACGTACCACTTCCCACACTGTCACTCAGAGTCTAAAGAAC K H S K T Y H F P K T V T Q S L K K T I	1210 1230 1250 CAGGTTCCCTCACAGCTTCACAATGTACAAATTGTATCAGAAGTTATTAGAAATGAT R F L H S F T M Y K L Y Q K L F L E M I	1270 1290 1310 AGGTAAACCAGGTCCAATCAGTAAAATAAGCTGCTTATAACTGGAAATGGCCATTGAGCT G N Q V Q S V K I S C L
1330 1350 GTTTCCTCACAAATTGGCGAGATCCCATGGATGATAA		

FIG. 9E

muoste0.frg	M N K W L C C A L L V L L D I E W T T Q E T I P P K Y L H Y D P E T G H Q L L C D K C A P G T Y L	50
ratoste0.frg	H N K W L C C A L L V F L D I E W T T Q E T I P P K Y L H Y D P E T G R Q L L C D K C A P G T Y L	50
huoste0.frg	H N K W L C C A L L V F L D I S I K W T T Q E T I P P K Y L H Y D E E T S H Q L L C D K C P P G T Y L	50
muoste0.frg	K Q H C T V R R K T I C V P C P D H S Y T D S H W H T S D E C V I C S P V C K E I Q S V K Q E C N R T	100
ratoste0.frg	K Q H C T V R R K T I C V P C P D Y S Y T D S H W H T S D E C V I C S P V C K E I Q T V K Q E C N R T	100
huoste0.frg	K Q H C T A K W K T V C A P C P D H Y T D S H W H T S D E C I Y C S P V C K E I Q V V K Q E C N R T	100
muoste0.frg	H N R V C E C E E G R Y L E I E F C I K H R S C P P G S G V V Q A G T P E R N T V C K K C P D G F F	150
ratoste0.frg	H N R V C E C E E G R Y L E I E F C I K H R S C P P G I G V I Q A G T P E R N T V C K K C P D G F F	150
huoste0.frg	H N R V C E C E K E G R Y L E I E F C I K H R S C P P G F G V V Q A G T P E R N T V C K K C P D G F F	150
muoste0.frg	S G E T S S K A P C I K H T N C S T F C I L L I Q K G N A T H D N V C S G N R E A T O K C G I D V T	200
ratoste0.frg	S G E T S S K A P C I K H T N C S S L G I L L I Q K G N A T H D N V C S G N R E A T Q N C G I D V T	200
huoste0.frg	S G E T S S K A P C I K H T N C S V E G I L L I T Q K G N A T H D N I C S G N S E S T Q K C G I D V T	200

FIG.9F

muosteo.frg	L C E E A F F R F A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R R H S S Q E Q T	250
ratosteo.frg	L C E E A F F R F A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R R H S S Q E Q T	250
huosteo.frg	L C E E A F F R F A V P T K F T P N W L S V L V D N L P G T K V N A E S V E R I K R Q H S S Q E Q T	250
muosteo.frg	F Q L K L W K H Q N R D Q E M V K K I I Q D I D L C E S S V Q R H L G H S N L T T E Q L L A L M E	300
ratosteo.frg	F Q L K L W K H Q N R D Q E M V K K I I Q D I D L C E S S V Q R H T G H A N L T T E Q L R I L M E	300
huosteo.frg	F Q L K L W K H Q N K D Q D I V K K I I Q D I D L C E N S V Q R H I G H A N L T F E Q L R S L M E	300
muosteo.frg	S L P G K K I S P E E I E R T R K T C K S S E Q L L K L L S L W R I K N G D Q D T L I K G L M Y A L K	350
ratosteo.frg	S L P G K K I S P D E I E R T R K T C K P S S E Q L L K L L S L W R I K N G D Q D T L K G L M Y A L K	350
huosteo.frg	S L P G K K V G A E D I E K T T K A C K P S D Q I L K K L L S L W R I K N G D Q D T L K G L M H A L K	350
muosteo.frg	H L K T S H F P K T V F H S L R K T M R F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
ratosteo.frg	H L K A Y H F P K T V F H S L R K T T I R F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
huosteo.frg	H S K T Y H F P K T V T Q S L R K T T I R F L H S F T M Y K L Y Q K L F L E M I G N Q V Q S V K I S C	400
muosteo.frg	L	401
ratosteo.frg	L	401
huosteo.frg	L	401

FIG. 10

ltmr C P Q - C K Y I H P O N N S I C C T K C H K G T Y L Y N D C P G P C O D T D C R E C E S G S F T A S 49
 humoste P P K Y L H Y D P E T S H Q D L C D K C P P G T Y L K Q H C T A K - W K T V C A P C P D H Y Y T D S 49

ltmr EN H L R H C L S C S - K C R K E M C O V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F 98
 humoste W H T S D E C L V C S P V C - K E L O Y U K - Q E C N R T H N R V C E C K K E C R Y L E I - - E - F 91

ltmr Q C F N C S L C L N G - T V H L S C Q E K Q N T V C T - C H A G F F L R E - - - N E C V S C 139
 humoste - C P K H R S C P P G F G Y V Q A G T P E R N T V C K R C P D G F F S N E T S S K A P C R K H 139

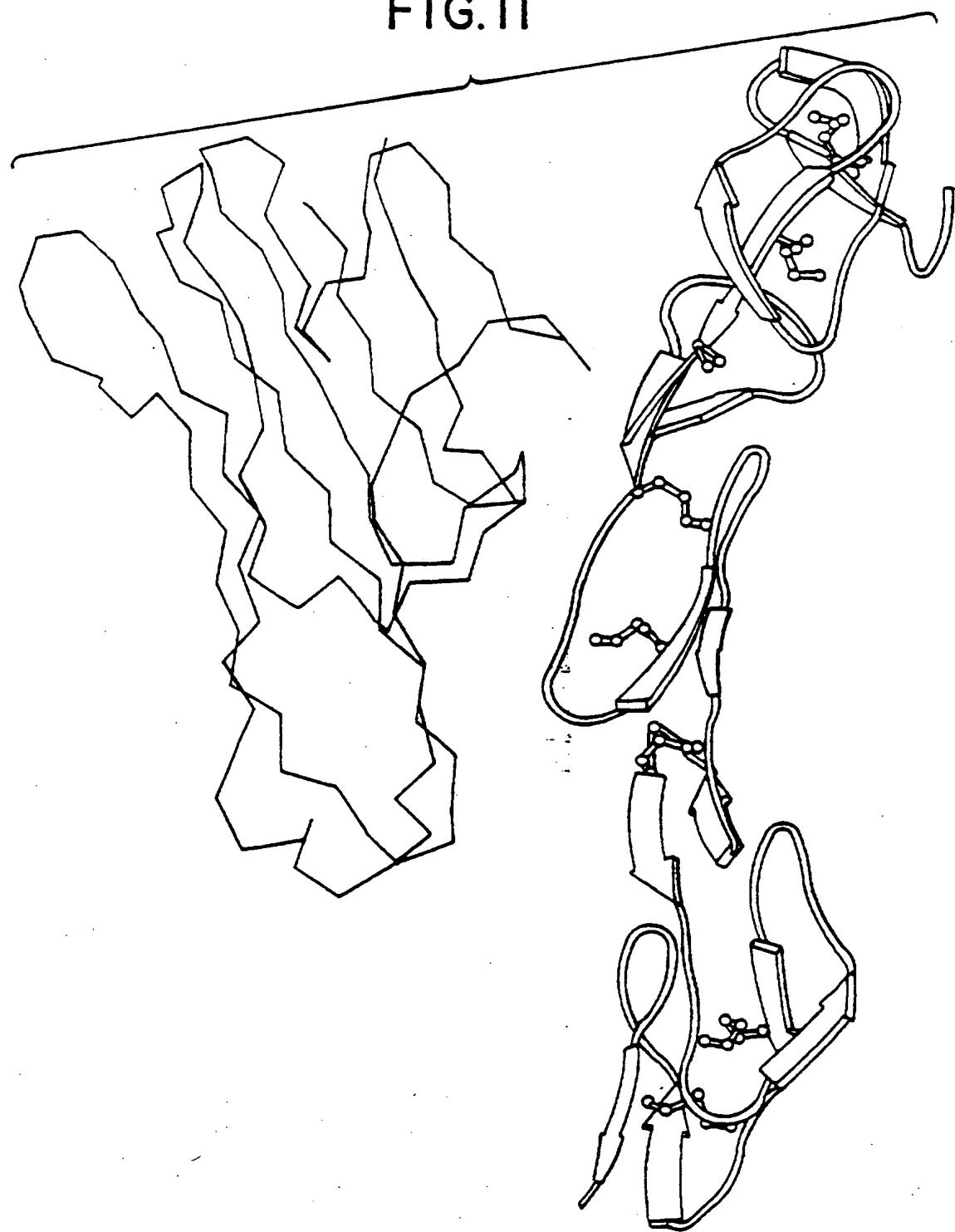
FIG. II

FIG. 12A

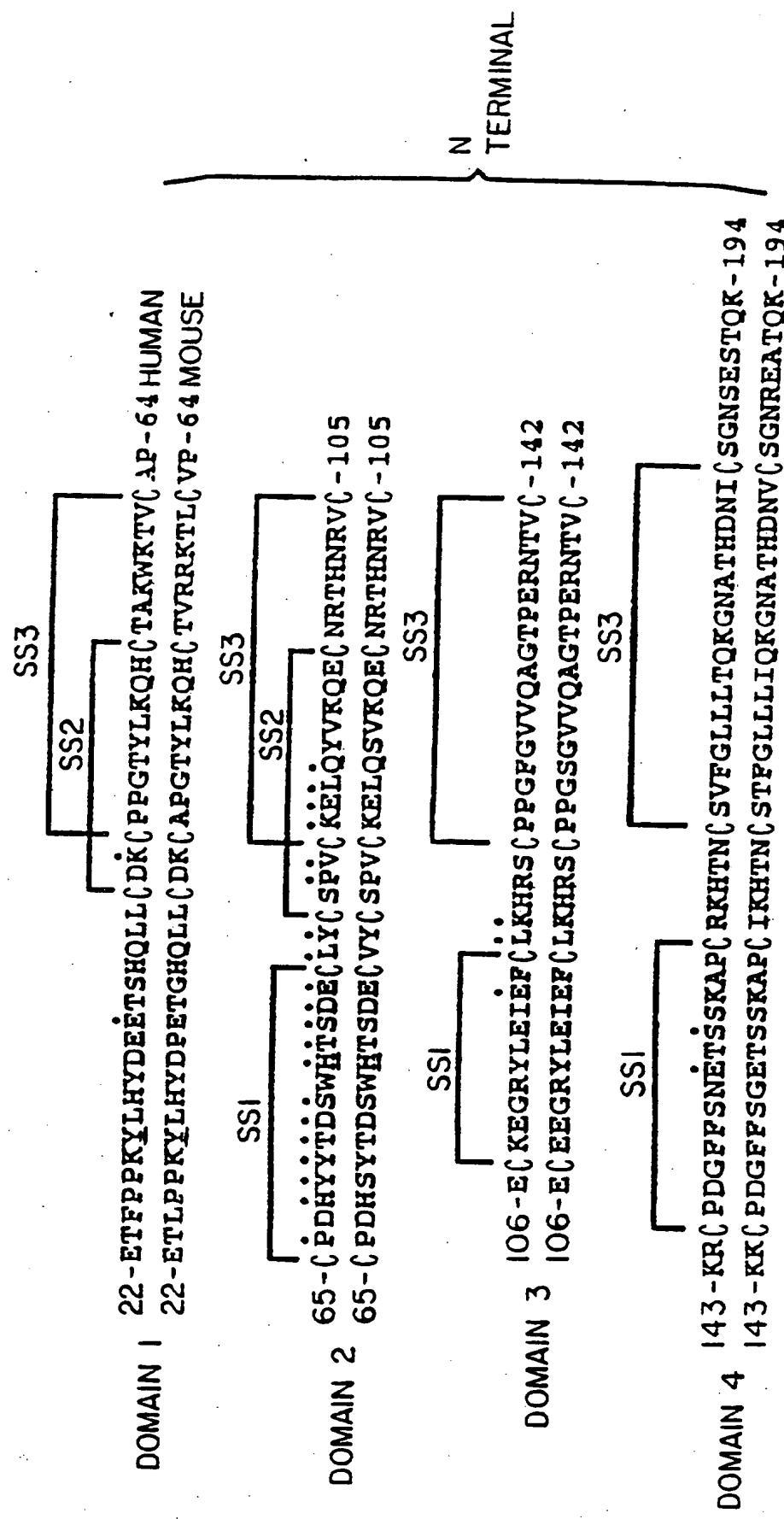


FIG. 12B

195 - C G ID V T L C E E A P F R F A V P T K P T P N W L S V L V D N L P G T K V N A E S V E R I K R Q H S S - 2 4 6
195 - C G ID V T L C E E A P F R F A V P T K I I P N W L S V L V D S L P G T K V N A E S V E R I K R R H S S - 2 4 6

247 - Q E Q T P Q L L K L W K H Q M K D Q D I V K K I I Q D I D C E N S V Q R H I G H A N L T P E Q L R S L - 2 9 8
247 - Q E Q T P Q L L K L W K H Q M R D Q E M V K K I I Q D I D C E S S V Q R H I G H S N L T T E Q L L A L - 2 9 8

C TERMINAL

299 - M E S L P G K K V G A E D I E K T I A C K P S D Q I L K L L S L W R I K N G D Q D T L K G L M H A L K - 3 5 0
299 - M E S L P G K K V S P E E I E R T R K T C K S S E Q L K L L S L W R I K N G D Q D T L K G L M Y A L K - 3 5 0

351 - H S K T Y H F P K T V T Q S L K K T I R P I H S E T M Y K L Y Q K L F L E M I G N Q V Q S V K I S C L - 4 0 1
351 - H L K T S H F P K T V T H S L R K T M R P I H S E T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C L - 4 0 1

2 8 / 4 6

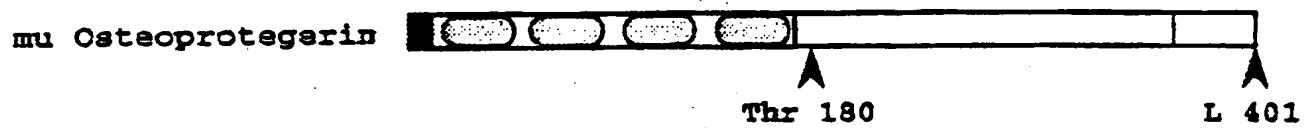
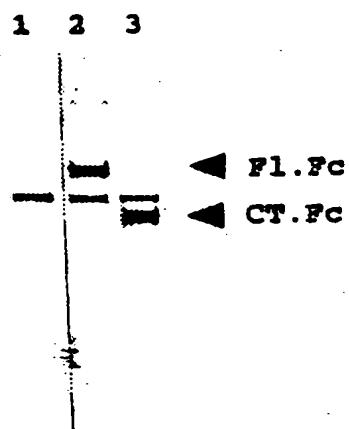
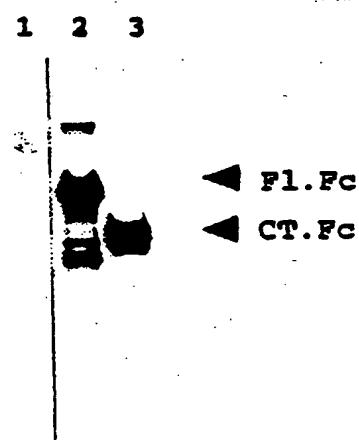
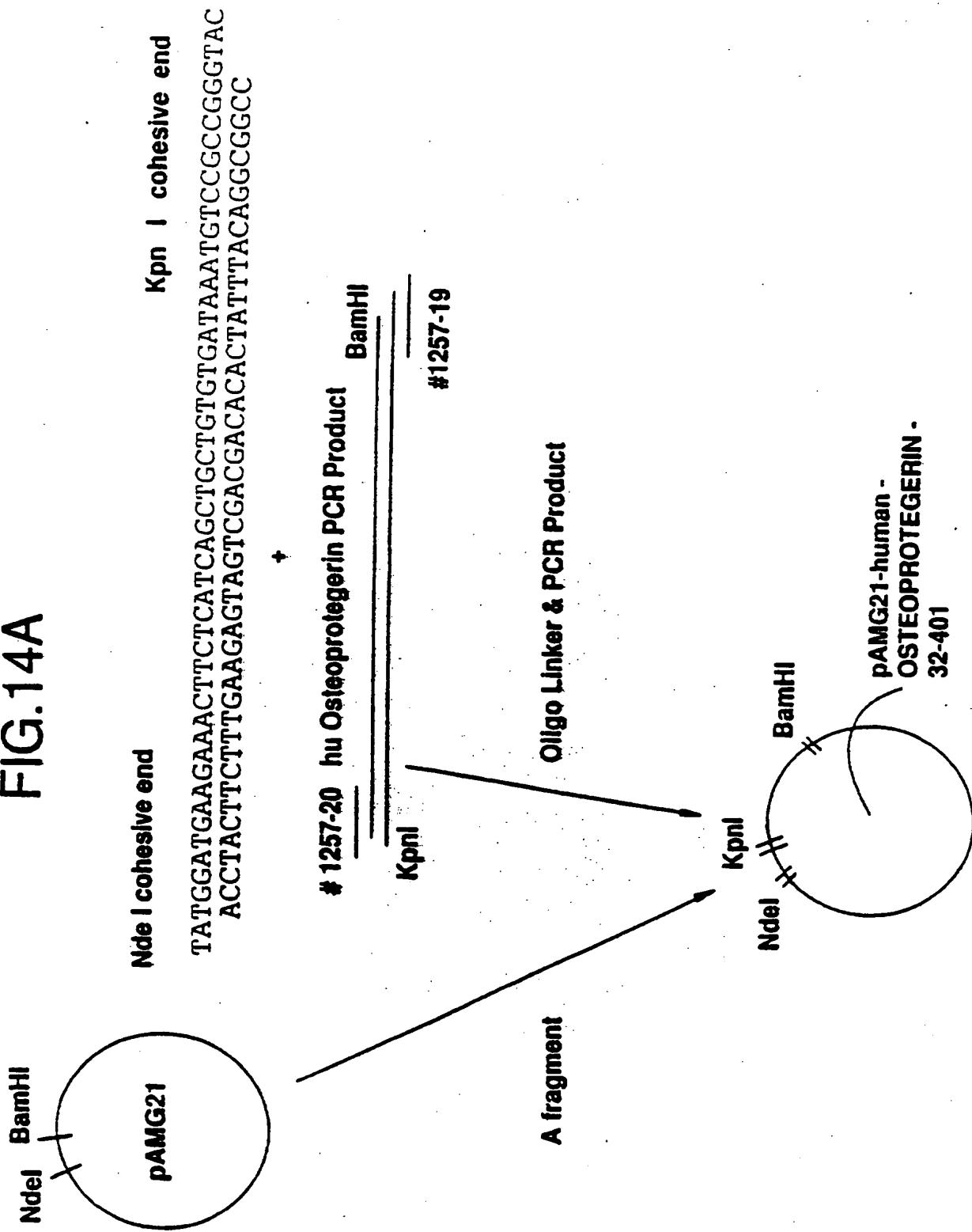
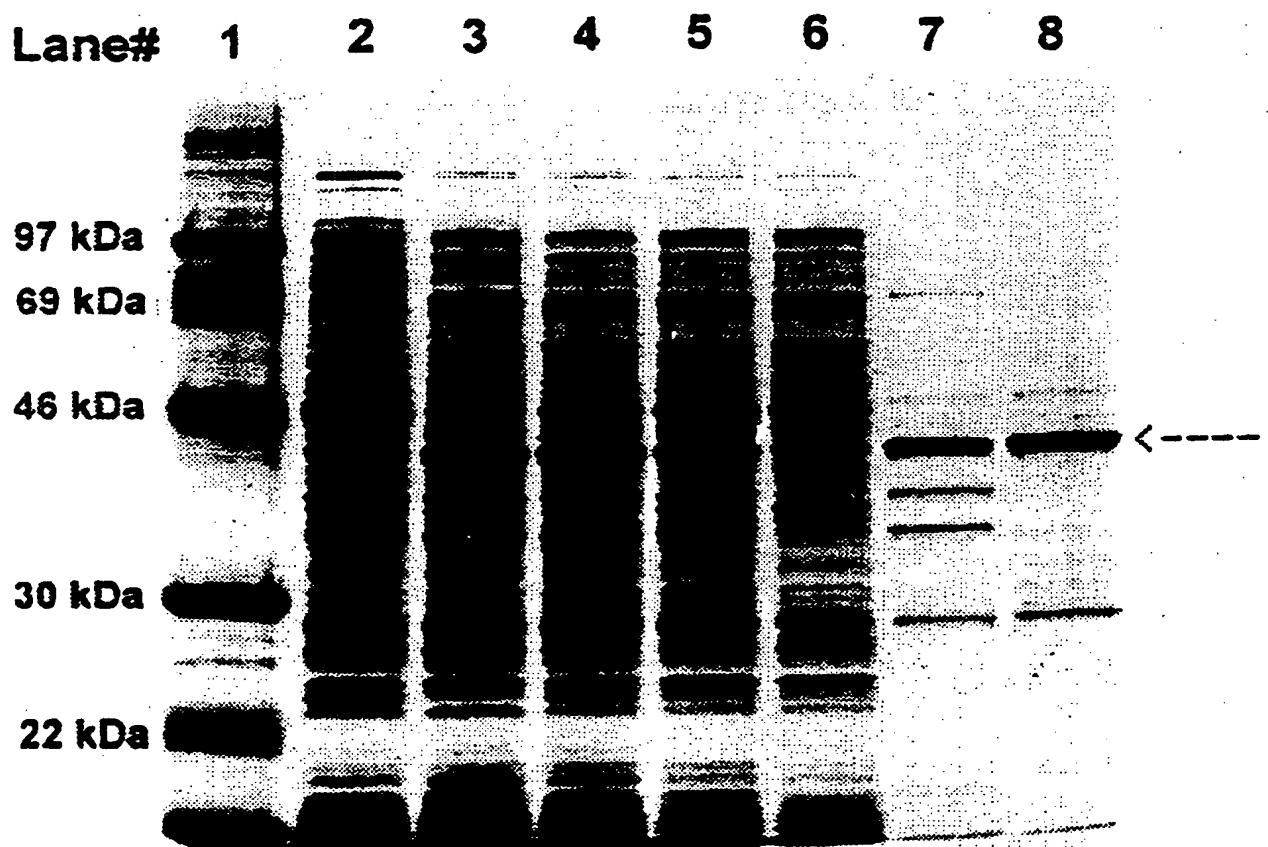
FIG.13A**FIG.13B****FIG.13C**

FIG. 14A

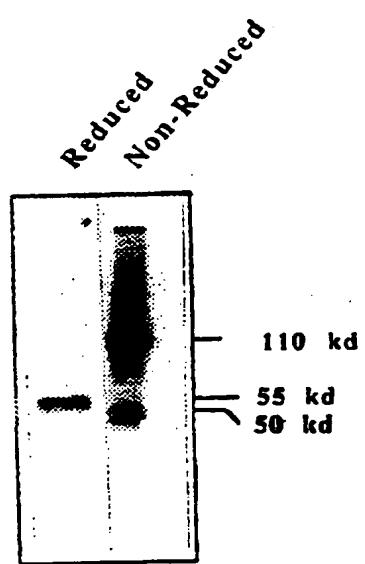


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FIG. 14B

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FIG. 15



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FIG. 16A

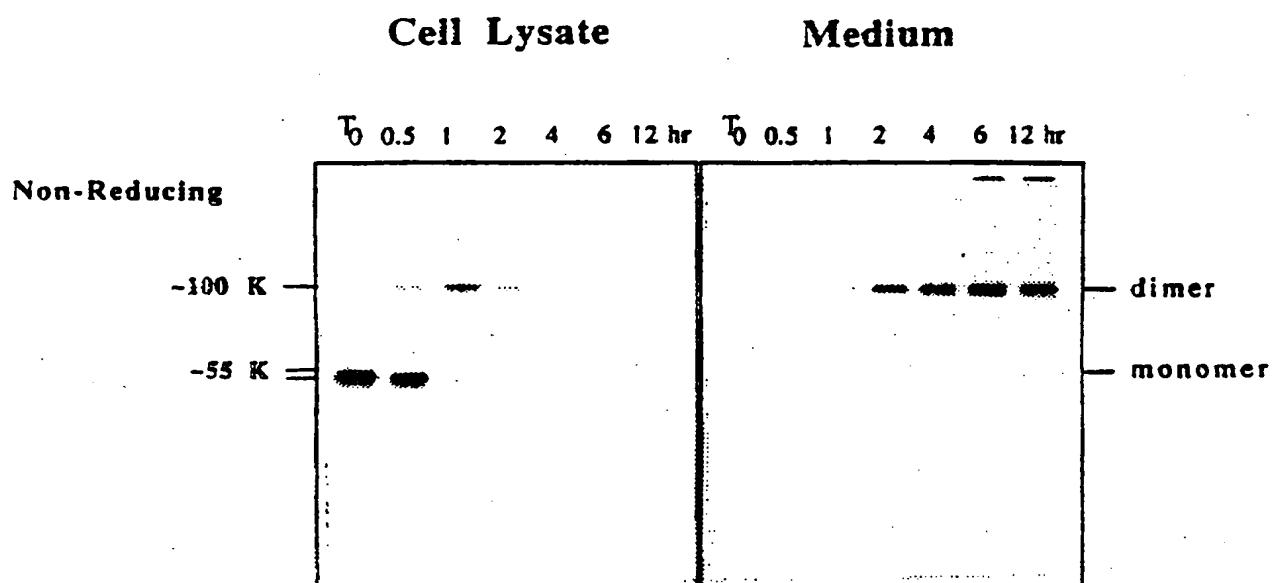
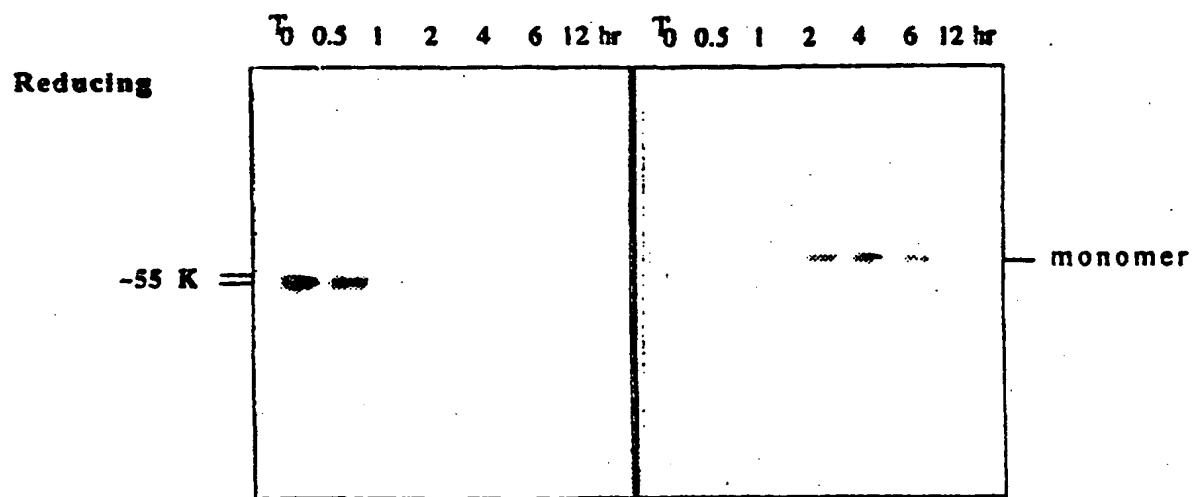
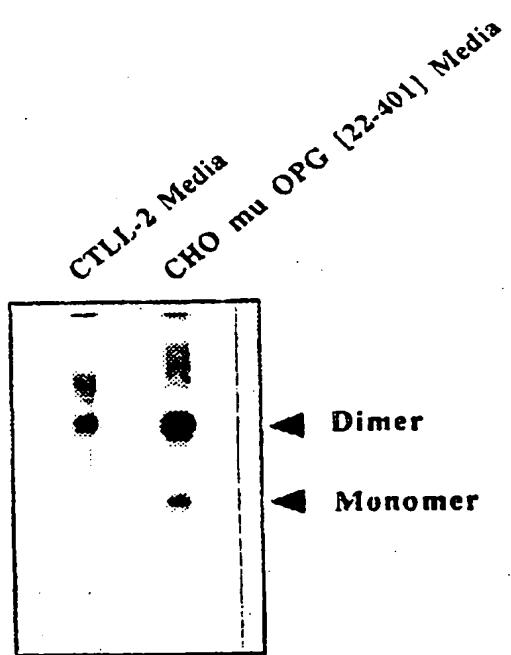


FIG. 16B



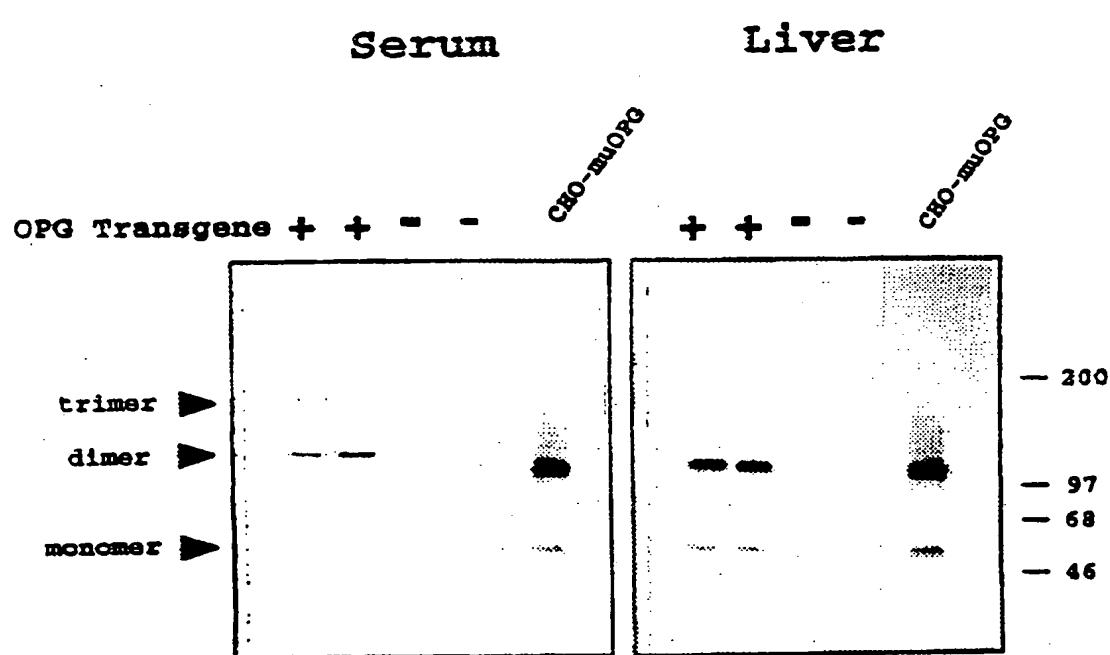
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FIG. 17



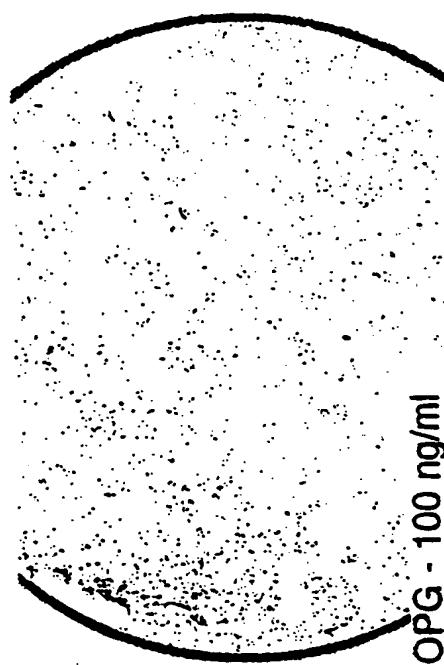
34 / 46

FIG. 18



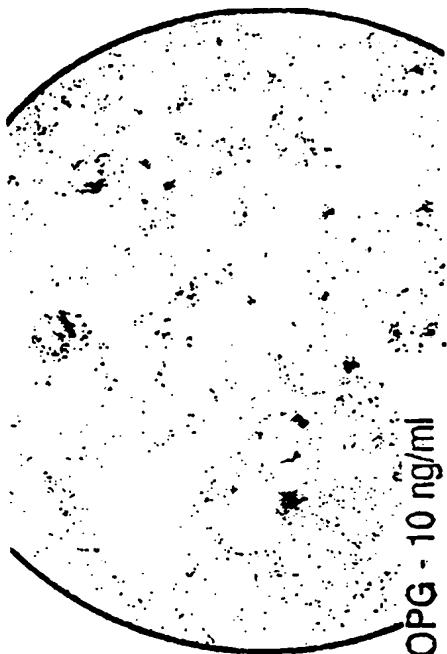
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FIG. 19A



OPG - 100 ng/ml

FIG. 19B



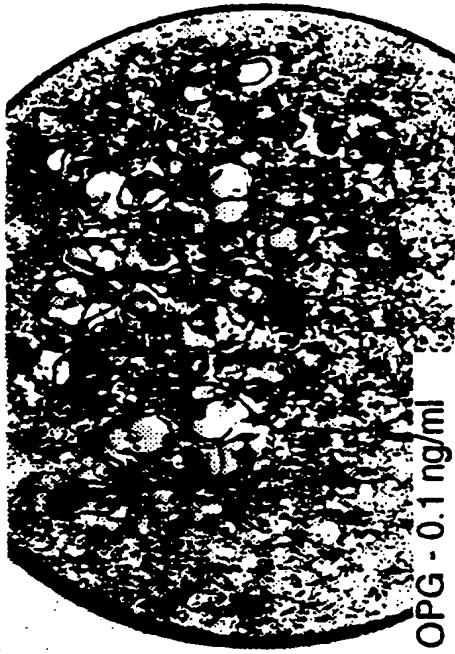
OPG - 10 ng/ml

FIG. 19C



OPG - 1 ng/ml

FIG. 19D



OPG - 0.1 ng/ml

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FIG. 19E

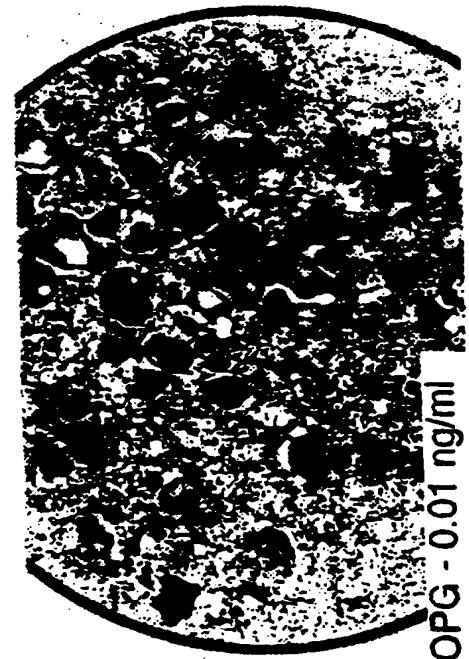
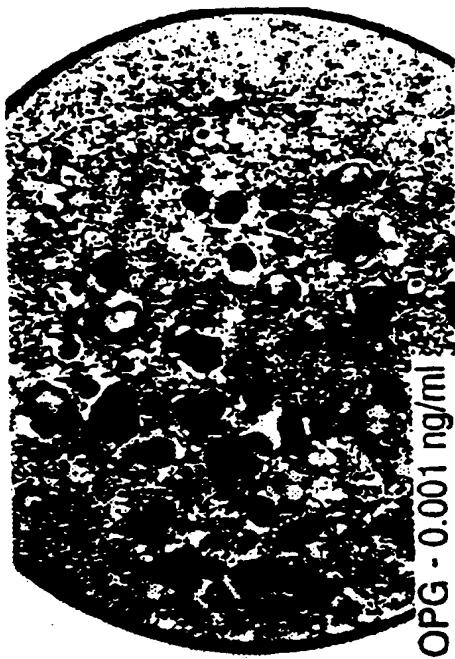


FIG. 19F



Control



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FIG.20

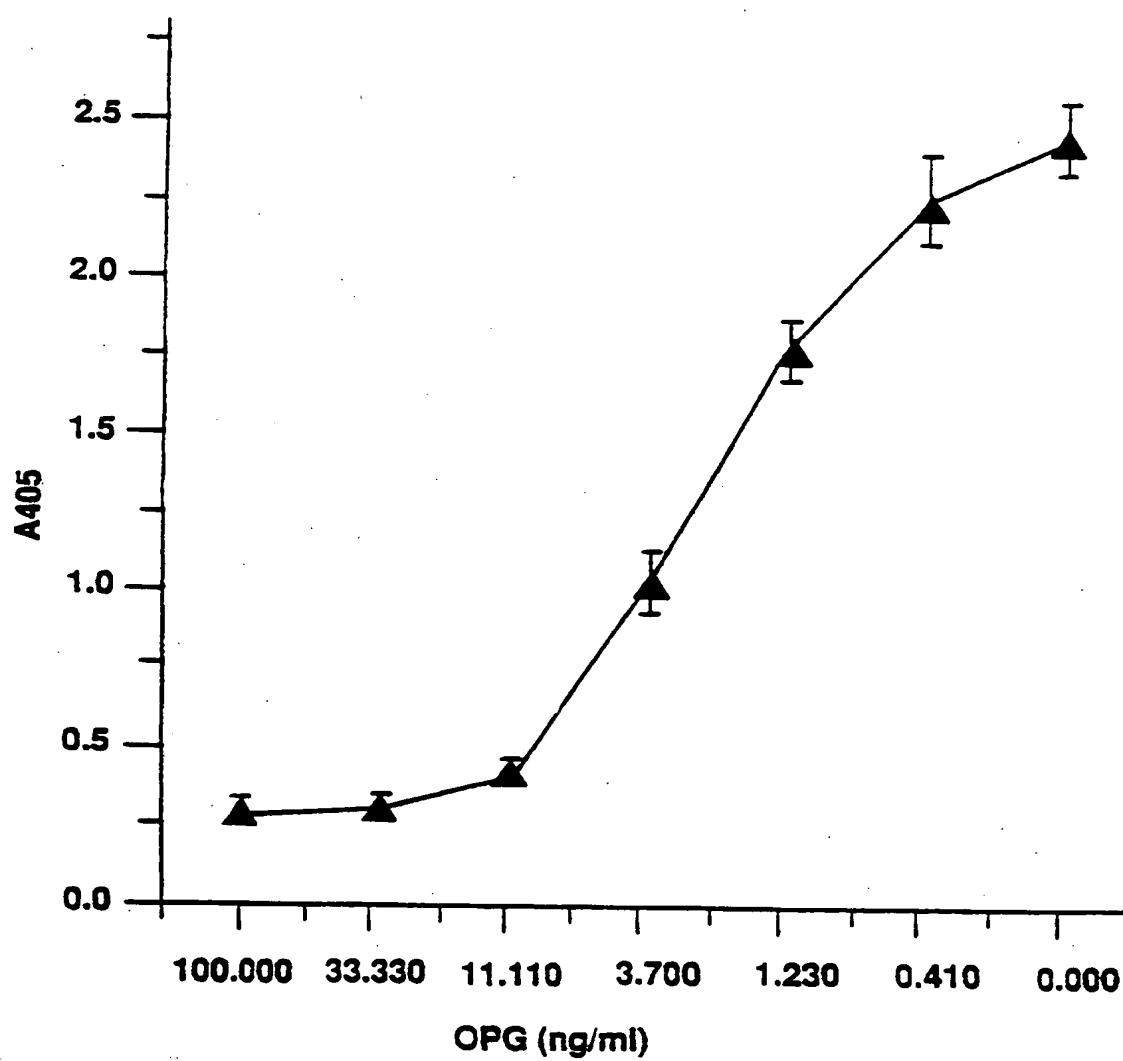
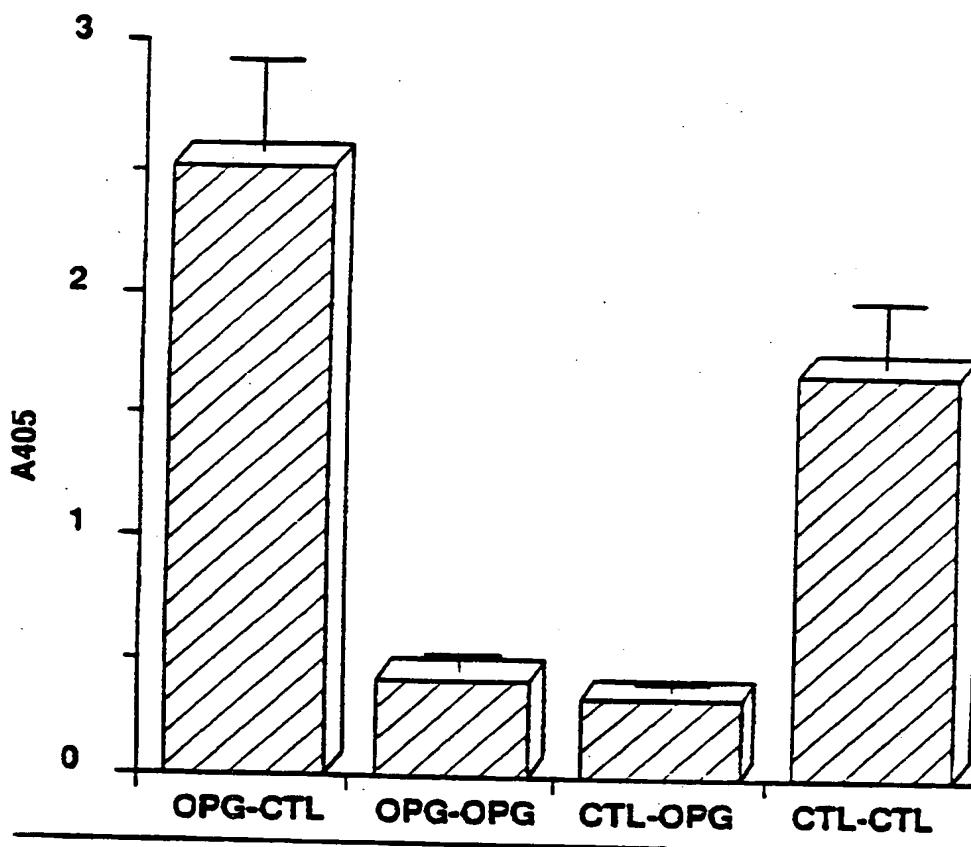


FIG.21

**Legend**

Growth
Bone marrow
cells
CSF-1

Intermediate
PGE2 + CSF-1

Terminal
ST2 cells
1,25(OH)2 D3
Dexamethasone

4 days

2 days

8 - 10 days

Groups

CTL - CTL

OPG - CTL

OPG - OPG

OPG - OPG

OPG

100 ng/ml

100 ng/ml

OPG

100 ng/ml

100 ng/ml

FIG.22A

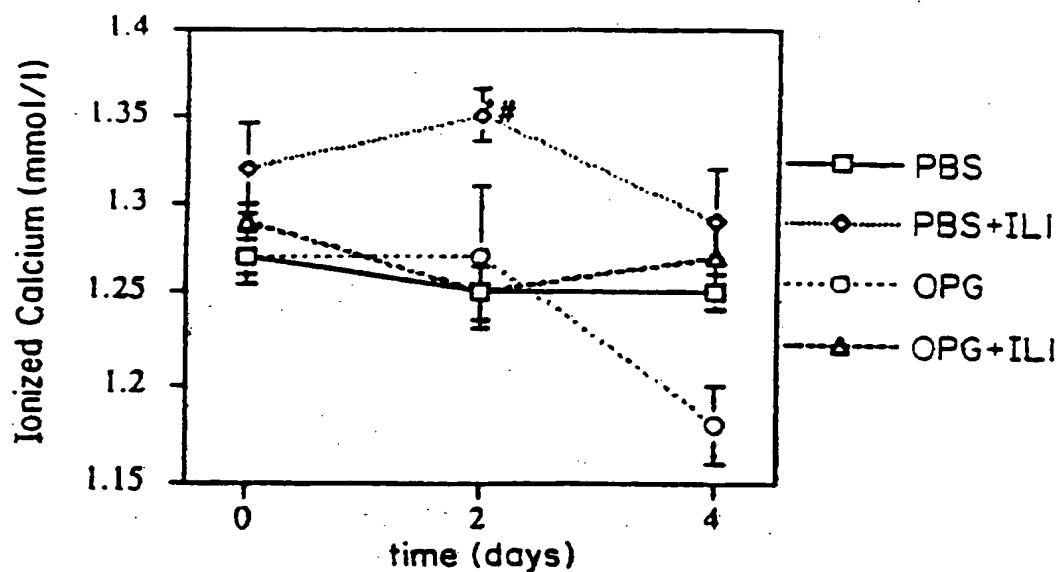
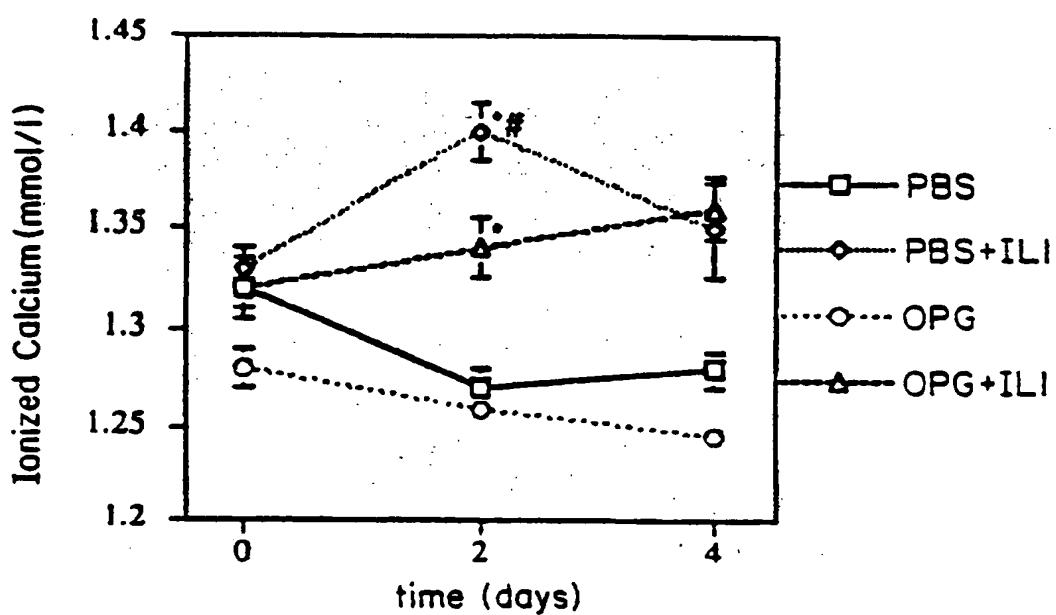


FIG.22B

* Different to PBS, $p < 0.05$ # Different to OPG + IL1, $p < 0.05$

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FIG.23A

PBS/PBS



FIG.23B



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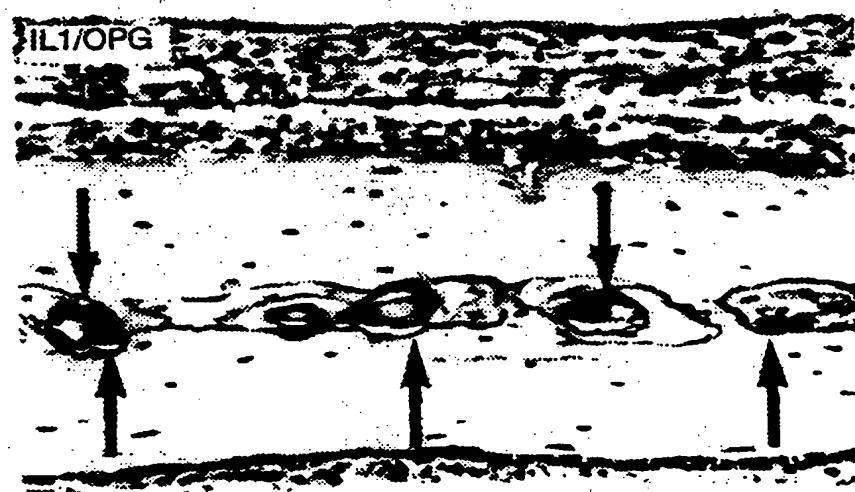
FIG.23C

PBS/OPG



FIG.23D

IL1/OPG



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FIG.24B
FIG.24A

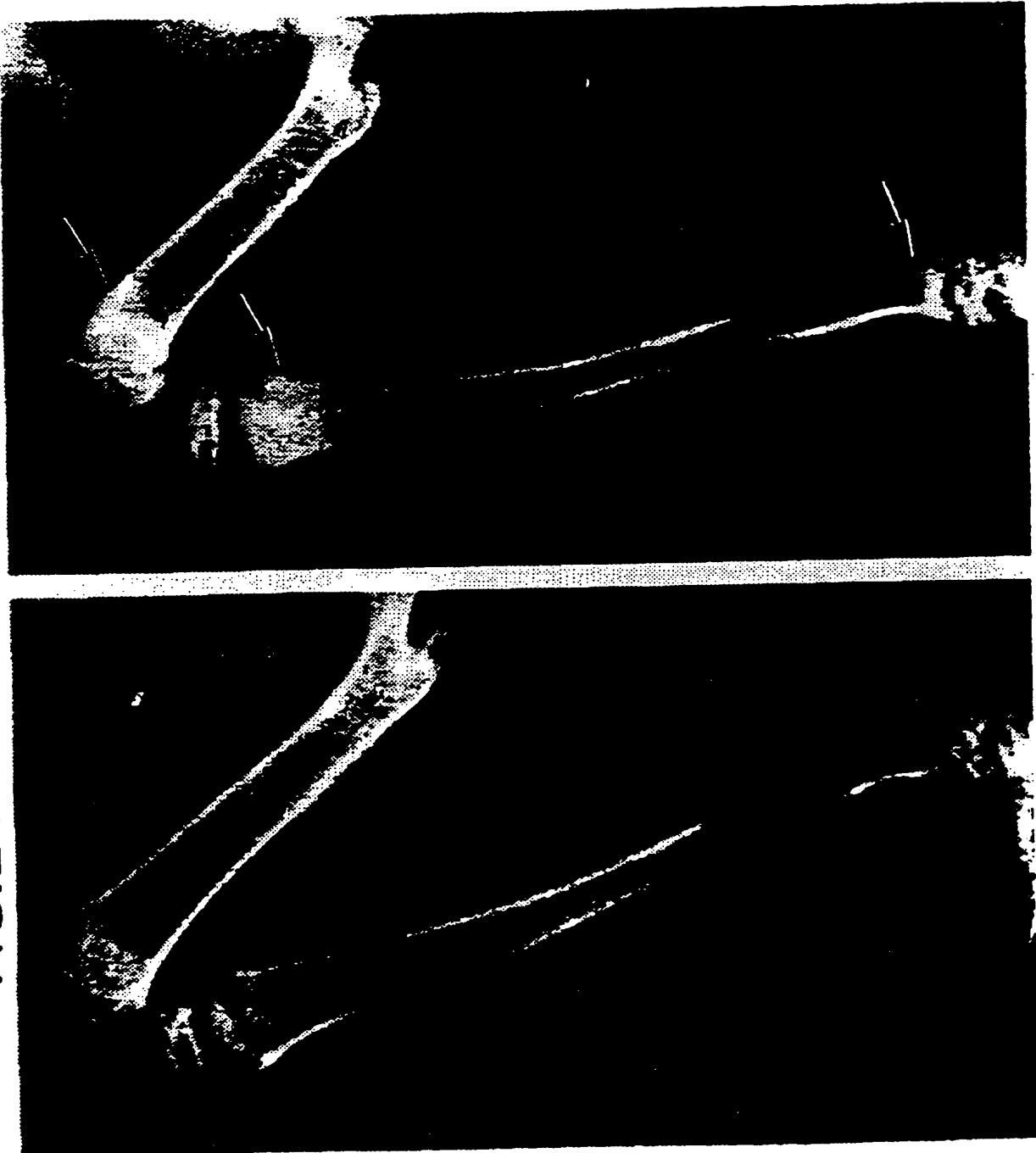
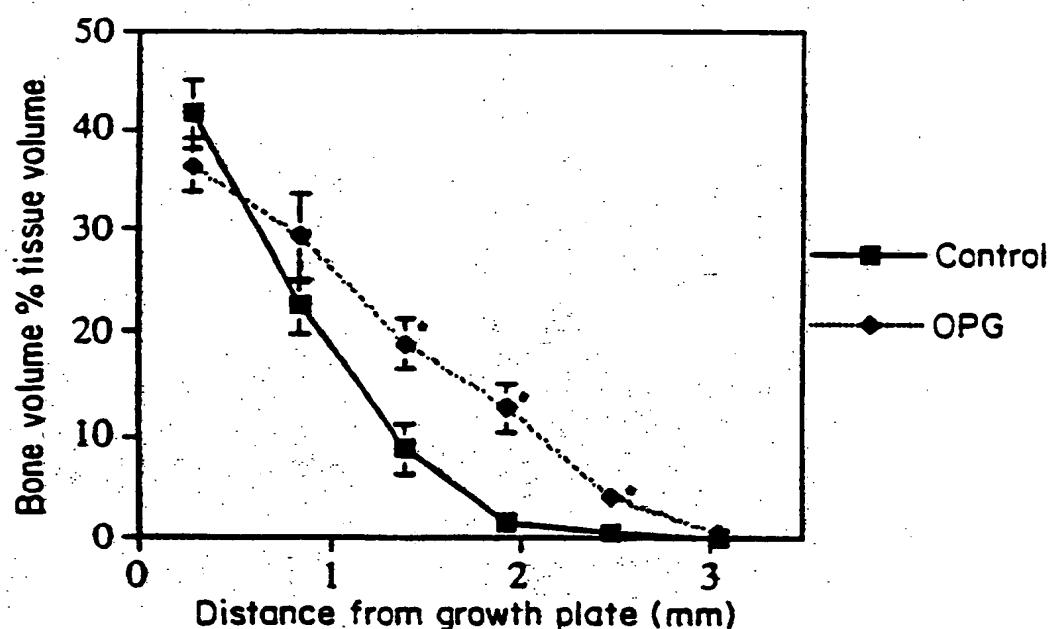


FIG.25



• Different to control $p < 0.01$

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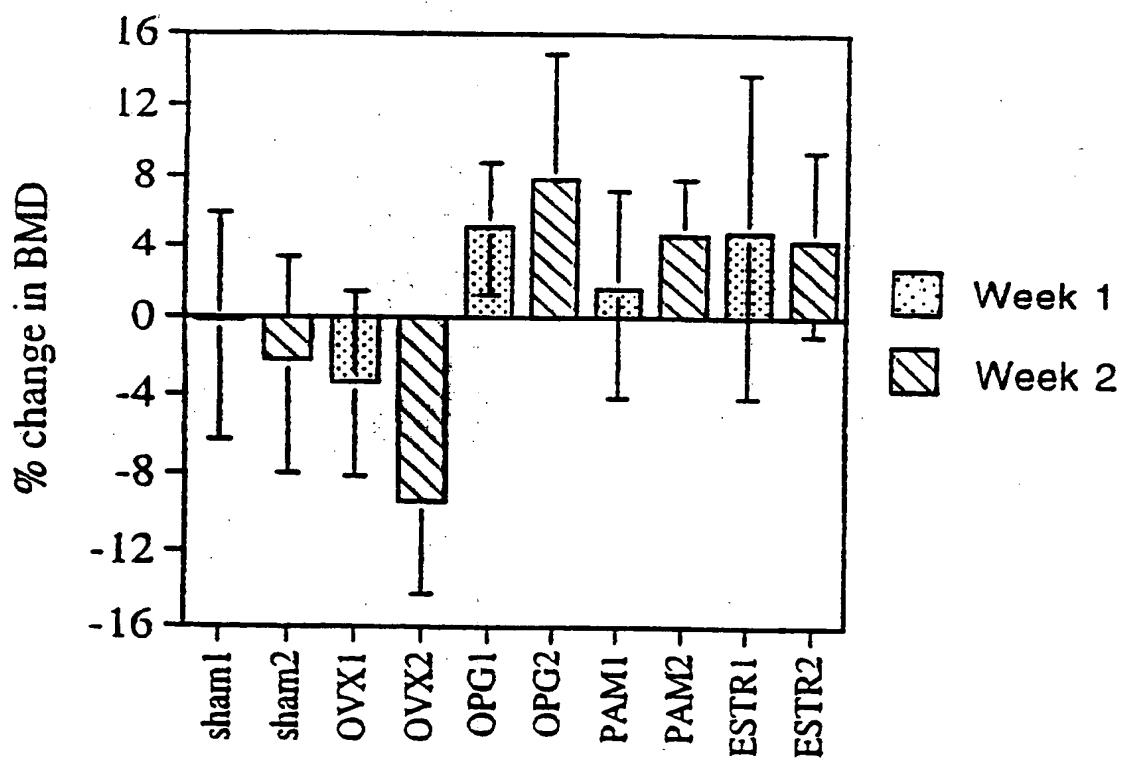
FIG.26A



FIG.26.B

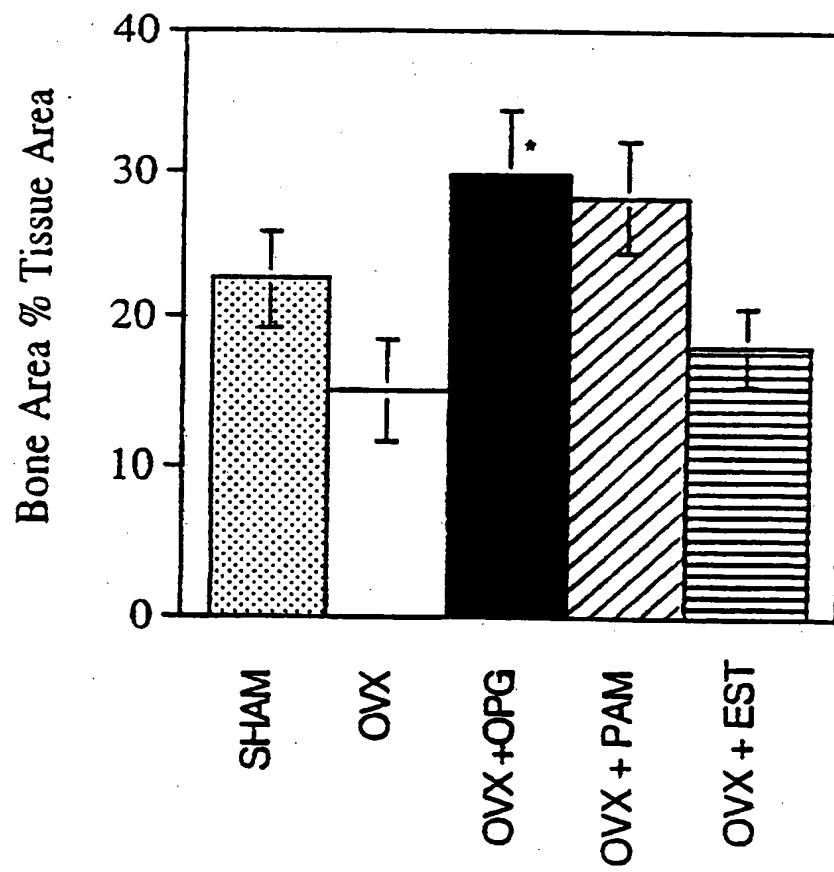


FIG.27



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FIG. 28



* Different to OVX $p < 0.05$

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/20621

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/715 C12N5/10 A01K67/027 C07K19/00
C12N15/62 C07K16/28 C07K1/107 C12Q1/68 G01N33/50
G01N33/566 A61K38/17 A61K48/00 C12N1/21 //((C12N1/21,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01K C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CELL, vol. 76, 25 March 1994, pages 959-962, XP002029050 SMITH C.A. ET AL.: "The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death." cited in the application see the whole document ---	1-60
A	PROC.NATL.ACAD.SCI.USA, vol. 88, 1991, pages 2830-2834, XP002029051 LEWIS M. ET AL.: "Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific." see figure 1 ---	1-60

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
9 April 1997	16. 04. 97

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentstaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.
Fax (+ 31-70) 340-3016

Authorized officer

Mandl, B

INTERNATIONAL SEARCH REPORT

Inte...al Application No.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol. 252, 1991, pages 1651-1656, XP000645049 ADAMS M.D.: "Complementary DNA sequencing:expressed sequence tags and human genome project." cited in the application see the whole document ---	1-60
A	US 5 447 851 A (BEUTLER BRUCE A ET AL) 5 September 1995 see the whole document ---	36-38,57
A	US 4 179 337 A (DAVIS FRANK F ET AL) 18 December 1979 cited in the application see the whole document -----	28,29

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'a' document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+ 31-70) 340-3016

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/20621

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 43-45, 49-53

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/20621

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5447851 A	05-09-95	NONE	
US 4179337 A	18-12-79	CA 1033673 A CH 616942 A DE 2433883 A FR 2313939 A GB 1469472 A JP 1152589 C JP 50042087 A JP 56023587 B NL 7409770 A SE 441753 B SE 7409366 A	27-06-78 30-04-80 05-02-76 07-01-77 06-04-77 30-06-83 16-04-75 01-06-81 22-01-75 04-11-85 21-01-75